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**The Role of Metabotropic Glutamate and Neurokinin Receptors in Mediating  
Sustained Nociceptive Inputs To The Spinal Cord of the Rat**

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**Thesis presented for the degree of Doctor of Philosophy**

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## **DECLARATION**

I hereby declare that the composition of this Thesis and the work presented in it are entirely my own with the exception of the electrophysiological experiments, which were carried out in collaboration with my supervisor, Dr S.M. Fleetwood-Walker. Some of the studies have been published, reprints of which are included in an appendix.

Marie R. Young

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## ABSTRACT

Glutamate, substance P (SP) and neurokinin A (NKA) are all found in fine primary afferent fibres and can be released upon noxious stimulation of the corresponding cutaneous receptive field. The possibility of a role in nociception for the metabotropic class of glutamate receptors (mGluRs) as well as those at which SP and NKA preferentially act (NK<sub>1</sub> and NK<sub>2</sub>), was investigated in the present study. Since protein kinase C (PKC) has been shown to be important in the mediation of noxious, but not non-nociceptive inputs, the potential role of this and several other signal transduction pathways in sensory inputs was assessed here, especially in the context of actions via mGluRs.

(a) Extracellular recordings were made from single dorsal horn neurons (laminae III-V) in the spinal cords of chloralose/urethane anaesthetised rats. Activity evoked by innocuous brushing of the cutaneous receptive field was not reduced by ionophoresis of mGluR antagonists L-1-amino-3-phosphopropionic acid (AP3), (R,S)- or (S)-4-carboxy-3-hydroxyphenylglycine (CHPG). However, sustained elevated firing evoked by the application of the C-fibre selective chemical irritant mustard oil was significantly attenuated by L (but not D)-AP3 and (R,S)-CHPG. Approximately one fifth of dorsal horn neurons could be excited by ionophoresis of the mGluR agonist (1S,3R)-1-aminocyclopentane-1-3-dicarboxylic acid (ACPD), and this evoked activity was similarly reduced by L (but not D)-AP3 and (R,S)-CHPG.

(b) Inhibitors of calmodulin/Ca<sup>++</sup>-dependent kinase (CamKII), phospholipase A<sub>2</sub> (PLA<sub>2</sub>), protein kinase A (PKA) and non-receptor tyrosine kinases were ionophoresed during brush- and mustard oil-evoked activity. CamKII and PLA<sub>2</sub> inhibitors were less effective in reducing innocuous rather than noxious inputs. Inhibitors of PKA and tyrosine kinases were equally effective in reducing all evoked activity. All of these antagonists, as well as two PKC inhibitors significantly decreased activity elicited by the ionophoresis of (1S,3R)-ACPD.

(c) The behaviour of conscious rats in response to noxious mechanical and thermal stimulation of one hindpaw (non-inflamed or inflamed by an intraplantar injection of carrageenan) was monitored following an intrathecal injection of mGluR antagonists L-AP3 and (S)-CHPG, as well as GR82334 and L659,874, antagonists at NK<sub>1</sub> and NK<sub>2</sub> receptors respectively. (S)-CHPG significantly increased withdrawal responses in each model to both mechanical and thermal stimulation, whereas L-AP3 was only effective in the inflamed paw. L659,874 was antinociceptive in thermal, but not mechanical, tests in the non-inflamed and inflamed paws. GR82334 did not alter latencies in any tests. However, a co-injection of GR82334 or L659,874 with

each mGluR antagonist resulted in an apparent reversal of the analgesic effects observed when the mGluR antagonists were administered alone.

This investigation demonstrates a role for spinal cord mGluRs in the transmission of sustained nociception, possibly mediated by PKC, CamKII and PLA<sub>2</sub>. NK<sub>2</sub> receptors appear to have a selective role in thermal inputs to the spinal cord, whereas this study provided no evidence for an overt role of NK<sub>1</sub> receptors in the nociceptive models assessed. It is possible that NK<sub>1</sub> receptors play a greater role in more prolonged or severe nociceptive inputs. The present data suggest however that not only NK<sub>2</sub>, but also NK<sub>1</sub> receptors exhibit a functional interaction with the influence of mGluRs on nociceptive thresholds.



## ABBREVIATIONS

$\alpha$ MeCPG	$\alpha$ -methyl-4-carboxyphenylglycine
AA	arachidonic acid
(1S,3R)-ACPD	(1S,3R)-1-aminocyclopentane-1-3-dicarboxylic acid
AMH	A $\delta$ fibre mechano-heat sensitive nociceptor
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AP-1	activator protein-1
APV	2-amino-5-phosphonic acid
ATP	adenosine triphosphate
Ca <sup>++</sup>	calcium
[Ca <sup>++</sup> ] <sub>i</sub>	intracellular calcium
CamKII	calmodulin/Ca <sup>++</sup> -dependent kinase
cAMP	cyclic AMP
CCI	chronic constrictive injury
cDNA	complementary deoxyribonucleic acid
Ci	Curie
CMH	C fibre mechano-heat sensitive nociceptor
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
COX	cyclooxygenase
CTZ	cyclothiazide
DAG	diacylglycerol
DEPC	diethyl pyrocarbonate
DRG	dorsal root ganglion
EAA	excitatory amino acid
GABA	$\gamma$ -amino butyric acid
GF109203X	2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)maleimide
GR82334	[D-Pro <sup>9</sup> [spiro- $\gamma$ -lactam]Leu <sup>10</sup> ,Trp <sup>11</sup> ]physalaemin(1-11)
[ <sup>3</sup> H]PDBu	[ <sup>3</sup> H]-phorbol-12,13-dibutyrate
HRP	horseradish peroxidase
IEG	immediate-early gene
IP <sub>3</sub>	inositol-1,4,5-triphosphate
IR	immunoreactive
ISSH	<i>in situ</i> hybridisation histochemistry
KA	kainate
L659,874	acetyl-Leu-Met-Gln-Trp-Phe-Gly-NH <sub>2</sub>
L (or D-)-AP3	L(or D)-1-amino-3-phosphopropionic acid
LI	like immunoreactivity
LTP	long-term potentiation
Mg <sup>++</sup>	magnesium
mg; $\mu$ g	milligram; microgram
mGluR	metabotropic glutamate receptor
MIA	mechanically-insensitive afferent
ml; $\mu$ l	millilitre; microlitre
mm; $\mu$ m	millimetre; micrometre

mRNA	messenger ribonucleic acid
nA	nanoamp
NGF	nerve growth factor
NK	neurokinin
NMDA	N-methyl-D-aspartate
nmol	nanomole
NO	nitric oxide
NOS	nitric oxide synthase
NSAID	non-steroidal anti-inflammatory drug
P	probability
PCR	polymerase chain reaction
PG	prostaglandin
PIP <sub>2</sub>	phosphatidylinositol-4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA <sub>2</sub>	phospholipase A <sub>2</sub>
PLC	phospholipase C
PPT	preprotachykinin
PSB	Pontamine Sky Blue
PSDC	post synaptic dorsal column
QA	quisqualate
R/s	response per second
ROI	region of interest
(S)- and (R,S)-CHPG	(S)- and (R,S)-4-carboxy-3-hydroxyphenylglycine
sem	standard error of mean
SMT	spinomesencephalic tract
SP	substance P
SRT	spinoreticular tract
SSC	sodium standard citrate
STT	spinothalamic tract
UV	ultra violet
VRP	ventral root potential

## CHAPTER 1: INTRODUCTION

*Pain is the perceptual counterpart of the body's response to stimuli that threaten the integrity of its tissues.*

(Treede *et al*, 1992).

### 1.1. GENERAL INTRODUCTION

In order to reach the brain somatosensory information is first relayed from peripheral receptors to the spinal cord (or in certain cases, the brainstem) by means of primary afferent fibres. Included in this barrage of information are signals contributing to the sensation of pain. During the past century there have been several major theories as to how painful (sensory) information is transferred to the central nervous system from the cutaneous surface of the body. These include the *intensity* theory (Goldscheider, 1884), which hypothesised that nociception occurred due to low-threshold receptors receiving a high enough intensity of stimulus to encode pain, and the *pattern* theory (Nafe, 1927), which claimed that a noxious stimulus gave rise to the appropriate pattern of discharges which was decoded centrally. The theory of *specificity* (Müller, 1842 and supported by Von Frey, 1895), differed markedly from the others in that it postulated the existence of receptors designed to selectively transmit painful information - nociceptors. Although this theory was correct in hypothesising a receptor responsible for nociception, it did not consider the variety of nociceptors, and their dynamic properties, that we are now familiar with.

#### 1.1.2. CUTANEOUS NOCICEPTION

There are various groups of primary afferent fibres, classified according to their size and conduction velocity, and whether they are myelinated or not. A $\alpha$  (group I) are the largest class of myelinated fibres and are found in muscle afferents, but not those innervating the skin. Non-noxious inputs from the cutaneous surface are mediated principally by large myelinated A $\beta$  (group II) fibres which have conduction velocities of greater than 30 metres per second (m/sec). Noxious inputs are mediated by fine afferent fibres, both myelinated A $\delta$  (group III) and unmyelinated C (group IV) fibres, with average conduction velocities of 5 - 30 m/sec and 0.5 - 2 m/sec respectively (Willis & Coggeshall, 1991).

Two types of receptors mediate sensory cutaneous inputs - corpuscle receptors and free nerve endings. The former include slowly and rapidly adapting touch receptors which mediate tactile sensations. In glabrous skin there are two types of rapidly adapting (Meissner's and Pacinian corpuscles) as well as two types of slowly adapting (Merkel receptor and Ruffini corpuscle) touch receptors. Hairy skin has all of these, with the exception of Meissner's corpuscle, and also contains hair follicle receptors. Some of these, type D hair follicles, are innervated by A $\delta$  fibres, therefore not all non-nociceptive inputs are A $\beta$  fibre-mediated. The free nerve ending group of cutaneous receptors includes nociceptors, of which there are several classes derived from both A $\delta$  and C fibres. Not all free nerve endings are exclusively nociceptive however, as warming and cooling of the skin is also detected by A $\delta$  and C fibres (Iggo, 1959; 1969). Since most nociceptors have been located in a manner whereby their responses to mechanical and heat stimuli have been characterised, they are often referred to as A and C mechano-heat sensitive fibres (AMH and CMH):

**(a) A $\delta$  Fibre Nociceptors:** Two types of AMH nociceptors have been reported. The first, found mainly in glabrous skin, responds to high-intensity thermal stimuli, typically greater than 53°C, as well as noxious mechanical stimuli (Adriansen *et al*, 1983; Torebjörk, 1985). The second type however is found only in hairy skin and is responsive to lower thresholds of mechanical/heat stimulation, as well as responding to chemical irritation (Adriansen *et al*, 1983). Around 50% of A $\delta$  nociceptors have very high mechanical thresholds, or do not respond to noxious mechanical stimuli at all, these are commonly referred to as mechanically-insensitive afferents (MIAs, Meyer *et al*, 1991; Davis *et al*, 1993).

**(b) C Fibre Nociceptors:** C mechano-heat nociceptors (also commonly known as C polymodal nociceptors) respond to high-intensity mechanical, heat (and cold) and chemical stimuli. The response characteristics of CMH receptors in a variety of species, including human, monkey and cat, are highly uniform (Bessou & Perl, 1969; Torebjörk, 1974; Beitel & Dubner, 1976; Kumazawa & Perl, 1977). Heat thresholds can range from 38°C to 50°C for these receptors (though they are typically above 41°C), with the response tending to depend on the previous experience of the unit (Torebjörk, 1993). Sensitisation, whereby a repeated electrical or natural stimulus evokes an increased response from the neuron, has been reported for C fibres, with up to ten minutes of no further stimulation being required for responses to return to control levels (Meyer *et al*, 1994). As well as polymodal C fibres, there

are also around 30% of C fibre nociceptors which belong to the MIA group (Handwerker *et al*, 1991; Meyer *et al*, 1991; Kress *et al*, 1992; Davis *et al*, 1993).

More recent studies have demonstrated that these MIAs, both A $\delta$  and C fibre nociceptors, respond to chemical irritation. Following sensitisation using chemical or electrical stimulation, many of these receptors will respond to previously suprathreshold mechanical and thermal stimuli, though some of them do appear to be thermo- or chemo-specific (Davis *et al*, 1993). By means of electrophysiological recording, either directly from the nerve, dorsal roots or central neurons, MIAs have been found in the skin of the rat, following continuous electrical stimulation (Handwerker *et al*, 1991; Kress *et al*, 1992), and the monkey after the application of an inflammatory 'soup' (Meyer *et al*, 1991; Davis *et al*, 1993), as well as in the knee joint of the cat subsequent to an intra-articular injection of kaolin/carrageenan (Schaible & Schmidt, 1985; 1988). In microneurography experiments, Torebjörk (1993) also identified such mechanically insensitive nociceptors in the human skin using topical application of mustard oil and/or capsaicin (both of which are known to irritate C fibres) to induce sensitisation.

Sometimes following such injury to the skin *hyperalgesia* is produced, whereby pain is experienced when normally subthreshold stimuli are used and the response to suprathreshold stimuli is exaggerated (Lewis, 1942; Hardy *et al*, 1952). Two forms of hyperalgesia exist: *primary* hyperalgesia to both mechanical and thermal stimuli, occurs at (or very close to) the site of injury and is thought to be due to both local and central mechanisms (Treede *et al*, 1992). *Secondary* hyperalgesia, which manifests itself as sensitisation to mechanical stimuli only, arises in an undamaged area around the injury, and is thought to come about through changes that predominantly occur in the spinal cord (see Treede *et al*, 1992 & Meyer *et al*, 1992).

From the periphery the nociceptor primary afferent extends to its cell body, the dorsal root ganglion (DRG) cell, before it enters the spinal cord by means of the dorsal roots. Anatomical tracing, using retrograde transport of several horseradish peroxidase (HRP) conjugates, and more recently a plant lectin (phaseolus vulgaris leuco-agglutinin; PHA-L) which reaches the end of fine fibres, along with electrophysiological recording experiments have shown that while the large A $\beta$  fibres enter the spinal cord from the medial portion of the dorsal roots to terminate in the deeper layers, the fine A $\delta$  and C afferents enter through the lateral portion, terminating predominantly in the superficial laminae (Light & Perl, 1979; McMahon & Wall, 1985; Sugiura *et al*, 1989). These axons do not have many collaterals and terminate in the spinal cord very close to the segment at which they enter

(Szentágothai, 1964). However, recent single fibre-labelling studies have highlighted the extensive branching which occurs once these fibres have entered the dorsal horn (Sugiura *et al*, 1986; 1989). Histological investigations as well as electrophysiological recording experiments have revealed that primary somatic afferent fibres terminate in a strict topographic pattern in the dorsal horn of the spinal cord (Szentágothai & Kiss, 1949; Brown & Fuchs, 1975; Koerber & Brown, 1980; Ygge & Grant, 1983; Swett & Woolf, 1985).

## **1.2. THE DORSAL HORN OF THE SPINAL CORD**

### **1.2.1. Classification of Sensory Neurons**

This study will refer to several types of dorsal horn neurons which can be classified according to their responsiveness to evoked sensory stimuli, (Iggo, 1974). *Non-nociceptive* (Class I) cells are innervated by sensitive mechanoreceptors and respond only to innocuous mechanical stimulation of the cutaneous receptive field (Dubner & Bennett, 1983). *Multireceptive* (Class II), also known as Wide-dynamic-range (WDR) neurons, have convergent inputs from peripheral nociceptors and sensitive mechanoreceptors, therefore respond to both innocuous and noxious mechanical stimulation of the skin as well as noxious thermal stimuli (Price *et al* 1976; 1978). Finally, *nocispecific* (Class III) neurons receive an exclusive input from cutaneous nociceptors and thus respond only to very high-threshold evoked stimuli (Cervero *et al*, 1976), both mechanical (Class IIIa) and mechanical and thermal (Class IIIb, Cervero & Iggo, 1980).

### **1.2.2. Laminar Organisation and Cutaneous Primary Afferent Input**

Anatomical variations throughout regions of the dorsal horn have been recognised since the last century. However, clear documentation of all areas was not duly recorded until 1952 when Rexed histologically examined the feline spinal cord and classified the laminae according to their cytoarchitectonic characteristics. It is Rexed's nomenclature that is most widely recognised today, and will be referred to throughout this study. Molander *et al* (1984) carried out a similar study on the rat spinal cord (see Figure 1.2) and found few differences between the two species. Within each species itself however, cytoarchitectonic organisation often differs slightly depending on the spinal segment (Rexed, 1952; Molander *et al*, 1984).



Where this occurs descriptions are given for lumbar segments, as those are of direct relevance to this study. Both investigations describe six layers in the dorsal horn:

(a) **Lamina I (The Marginal Zone):** This, the thinnest and most superficial layer, partially follows the lateral edge of the dorsal horn. The most prominent neurons of lamina I, marginal cells (Waldeyer, 1888), have dendritic trees which run mediolaterally within this layer. As well as these cells, lamina I contains other cells which merge with the outer layer of lamina II (Lima & Coimbra, 1983) and many nerve fibres. Innervation includes dorsal root afferents (A $\delta$ ) and axons of deeper dorsal horn units, including C fibres from lamina II (Cervero & Iggo, 1980; see Figure 1.2). Using HRP labelling, Gobel *et al* (1980) claimed that some fine (C) afferent fibres directly enter lamina I. Electrophysiological recording experiments in cats first documented nociceptive cells in this lamina (Christensen & Perl, 1970) and this was later confirmed by other studies in the monkey and rat (see Cervero & Iggo, 1980). Wide dynamic range units have also been found in lamina I (Christensen & Perl, 1970; Handwerker *et al*, 1975a; Cervero *et al*, 1976; Men  trety *et al*, 1977; R  thelyi *et al*, 1983).

(b) **Lamina II (The Substantia Gelatinosa):** As with lamina I, this layer also curves around the lateral edge of the dorsal horn (see Figure 1.1). Due to its gelatinous appearance, probably because of the large number of small cells and unmyelinated afferent fibres contained there (Szent  gothai, 1964; Light & Perl, 1979), the term 'substantia gelatinosa' was given to this region by Rolando (1824). It was further divided into two areas by Rexed (1952), the outer (most dorsal) and inner layers (see Figure 1.2). Ramon y Cahal (1909) classified two types of neurons in this region, both of which are among the smallest found in the spinal cord: the *limiting* (or *stalked*, Gobel, 1975; 1978) cells are situated in the outer region (cell bodies 10-15  m), whereas *central* (or *islet*, Gobel, 1975; 1978) cells, which are slightly smaller (7-14  m) and more compact, are found in the inner portion. Both cells have dendritic trees and axon collaterals which remain almost completely within the superficial layers (I-III) of the dorsal horn, though their mediolateral and rostrocaudal branching is widespread (Cervero & Iggo, 1980). A combination of degeneration and Golgi experiments have demonstrated that many C afferent fibres terminate in this lamina (LaMotte, 1977; R  thelyi, 1977; Ralston & Ralston, 1979), with PHA-L injection of single fibres recently confirming this (Sugiyara *et al*, 1989). The termination of A $\delta$  nociceptors in this lamina is very sparse (Willis & Coggeshall, 1991).

## Figure 1.1.

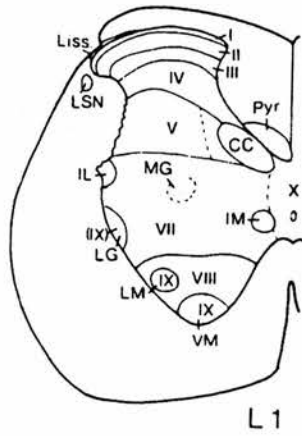
### Schematic drawings of the cytoarchitectonic subdivisions of segments L1 - L6 of the rat spinal cord

The laminar divisions of Rexed (1952) can be seen re-presented here in the rat spinal cord. The dorsal horn consists of laminae I - VI.

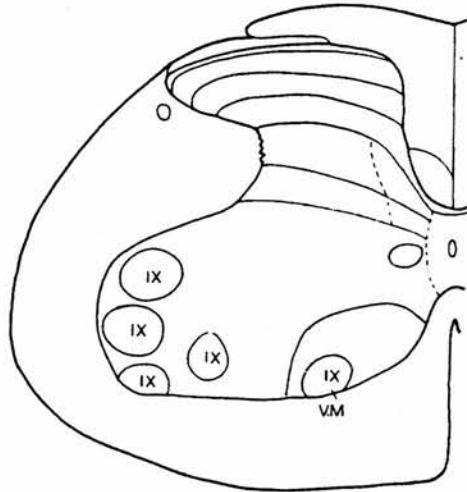
**CC** - column of Clarke; **IL** - intermedio-lateral nucleus; **IM** - intermedio-medial nucleus; **LSN** - lateral spinal nucleus; **Liss** - Lissauer's tract; **LG** - lateral group of large cells in the dorso-lateral part of the ventral horn; **LM** - latero-medial nucleus; **MG** - medial group of large neurons in the intermediate zone.

(Taken from Molander *et al*, 1984)

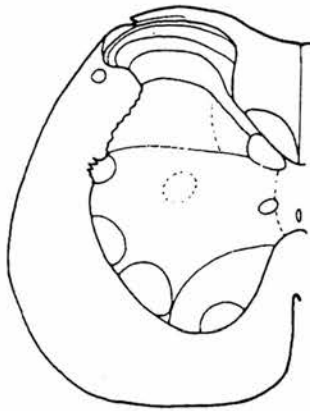




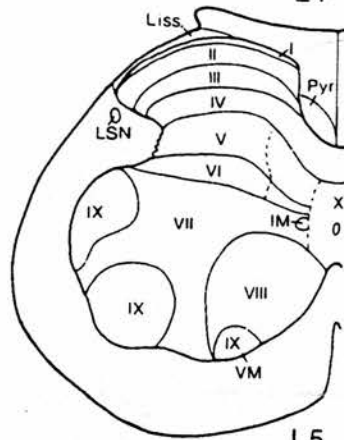
L1



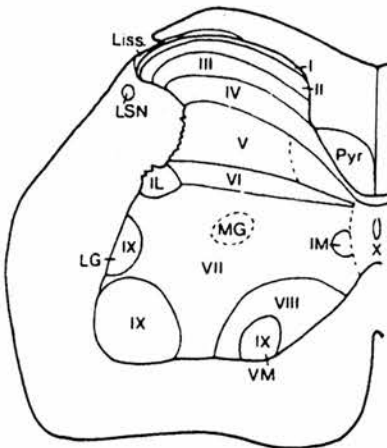
L4



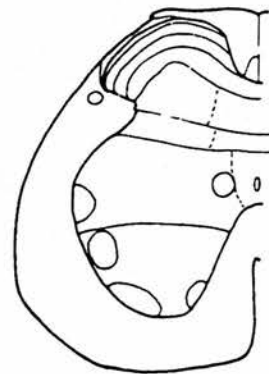
L2



L5



L3



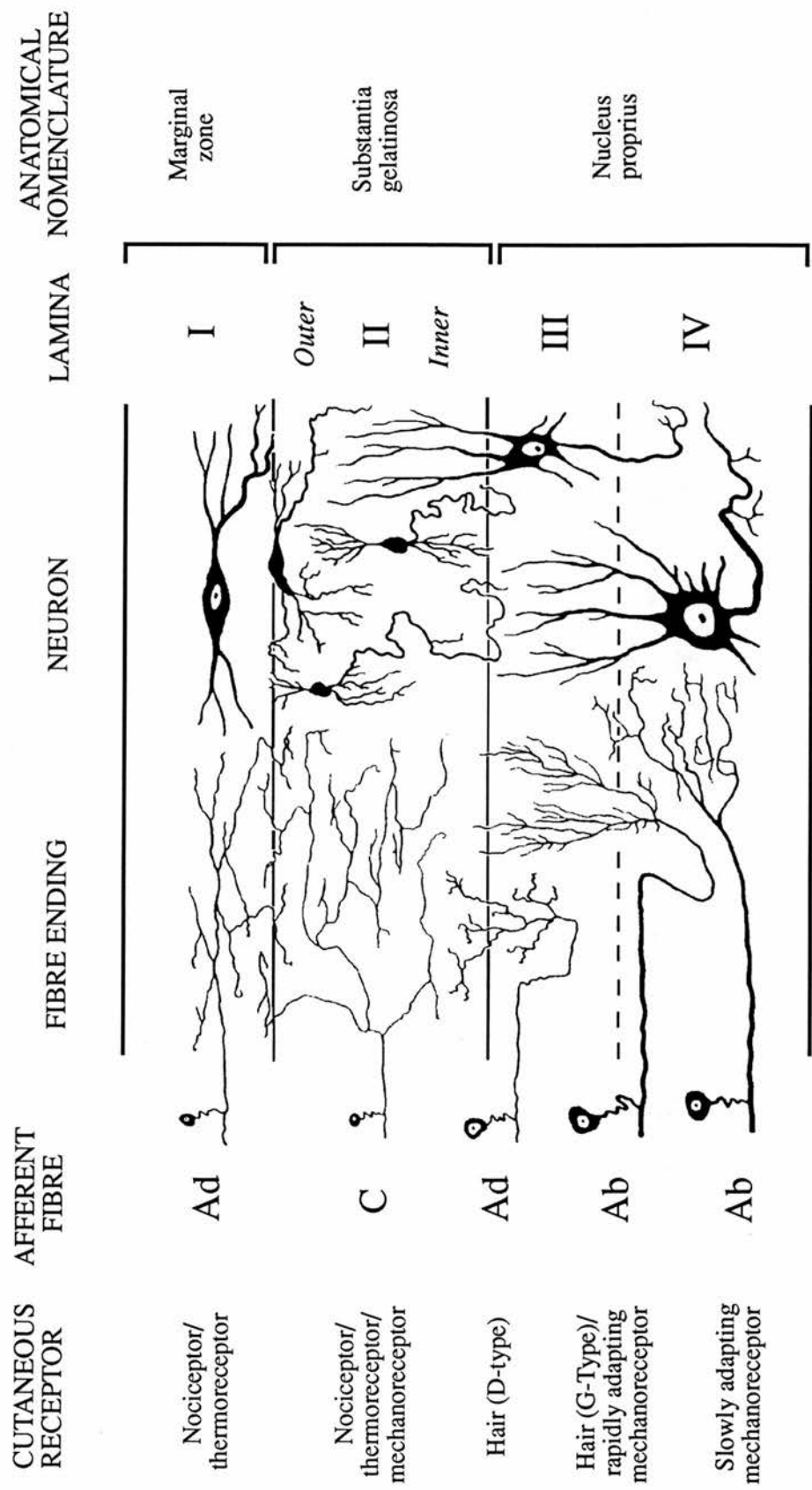
L6

## Figure 1.2.

### **Schematic diagram of the neuronal organisation of the superficial dorsal horn and its afferent input**

A hypothetical transverse section of the dorsal horn is represented, showing the types of neurons and afferent fibres which terminate there. The receptors which are associated with each fibre type are listed on the left of the diagram. Indicated on the right are the laminar divisions of Rexed (1952), along with the corresponding anatomical nomenclature. The neurons schematised are typical of those found in each lamina: (*from top to bottom*) a marginal cell, an SG limiting cell, two SG central cells and two neurons of the nucleus proprius, the most superficial of which has dendrites penetrating lamina II.

(Taken from Cervero & Iggo, 1980)



As a consequence of the very small size of lamina II cells there have been relatively few electrophysiological experiments recording directly from them. Nevertheless, activity of presumed lamina II cells in monkeys and cats (defined either by depth/position and/or intracellular HRP staining) has been recorded, both intra- and extra-cellularly and despite much controversy these studies have revealed several common features (Kumazawa & Perl, 1976; 1978; Cervero *et al*, 1977a; 1977b; Wall 1978; Light & Perl, 1979; see also Cervero & Iggo, 1980). Firstly, all investigations found that substantia gelatinosa cells are highly responsive to activity in cutaneous C afferent fibres. Secondly, lamina II cells demonstrated habituation to repeated stimulation of the cutaneous surface. Thirdly, neurons in this lamina, of all classes, could be inhibited by cutaneous (innocuous, noxious or both) stimuli. Although A $\beta$  fibre-evoked inputs have been reported to excite substantia gelatinosa cells, this is, on the basis of latency measurements, thought to be polysynaptic in nature (Price *et al*, 1979; Wall *et al*, 1979; Fitzgerald, 1981).

**(c) Laminae III - IV (The Nucleus Proprius):** Lamina III is sometimes thought of as a transitional layer between the substantia gelatinosa and the remainder of the dorsal horn, with the II/III border sometimes hard to distinguish (Molander *et al*, 1984). Lamina III contains many small cells, whereas neuronal size in layer IV is more heterogeneous, including scattered large neurons (Rexed, 1952; Molander, 1984). Lamina III receives direct inputs from both A $\delta$  and A $\beta$  fibres (Brown *et al*, 1977; Light & Perl, 1979), though many of the A $\delta$  fibres are of D type hair follicle receptor in origin. The input to lamina IV is from larger fibres, however many of these project their dendrites superficially to laminae I - III, therefore receiving direct inputs from these layers (Willis & Coggeshall, 1991). Consequently, many of the neurons in these laminae are multireceptive, with some studies also reporting nocispecific neurons here (Kumazawa & Perl, 1977; Men  trety *et al*, 1977).

**(d) Laminae V - VI:** This layer contains fewer neurons than those superficial to it and they are larger still (Rexed, 1952; Molander *et al*, 1984). Some nociceptive (A $\delta$ ) afferents do terminate in layer V and both nocispecific and multireceptive neurons are found in this region (Kumazawa & Perl, 1977; Men  trety *et al*, 1977; R  thelyi *et al*, 1982). The final lamina of the dorsal horn, layer VI, has a slightly curved ventral edge. Similar to lamina V, there are large cells, but layer VI

also contains smaller units in its medial portion (Rexed, 1952; Molander *et al*, 1984). Both of these layers receive direct inputs from large A $\beta$  afferent fibres (Molander *et al*, (1984).

There are in total ten laminae described by Rexed (1952) and Molander *et al* (1984), but the remainder are principally ventral horn structures and are beyond the scope of this study.

### **1.2.3. Descending Inputs to the Dorsal Horn**

As well as primary afferent inputs from peripheral areas, spinal cord neurons receive efferent input from descending fibres. These pathways are important in the maintenance of inhibitory control on dorsal horn neurons. Several areas in the brainstem, including the periaqueductal grey, the raphe nuclei, reticular nuclei and the locus coeruleus, provide analgesia when stimulated, (Reynolds, 1969; Oliveras *et al*, 1974; Segal & Sandberg, 1977). Moreover, when these supraspinal sites are stimulated the result is often, though not always, a specific inhibition of activity evoked by noxious (mechanical, thermal and electrical), and not non-noxious stimuli applied to the periphery or peripheral nerves (Oliveras *et al*, 1974; Willis *et al*, 1977; Carstens *et al*, 1979; Duggan & Griersmith, 1979; Hodge *et al*, 1981; Yeziarski *et al*, 1982; Gebhart *et al*, 1983; Morton *et al* 1983; Mokha *et al*, 1985). Various transmitters are associated with such inhibitory effects, including the opioid peptides, serotonin (5-HT), noradrenaline, dopamine and the inhibitory amino acids  $\gamma$ -aminobutyric acid (GABA) and glycine (see Besson & Chaouch, 1987 for review). Electrophysiological studies utilising reversible cold blocking of the spinal cord have demonstrated that there is a tonically active descending inhibitory influence on dorsal horn cells (Wall, 1967; Brown, 1971; Handwerker *et al*, 1975b; Duggan *et al*, 1977). These and other experiments have shown that neurons in lamina I and IV-VI are under such influences (Besson *et al*, 1975; Cervero *et al*, 1976; Duggan *et al*, 1981), though no evidence supports such tonic inhibition on lamina II units (Cervero *et al*, 1979). Tonic inhibitory effects are not reduced by the administration of naloxone, an opioid antagonist, and mixed effects of monoamine antagonists have been reported (see Duggan, 1985). The exact origin of this tonic inhibition is unclear, though bilateral lesions of the lateral reticular nuclei and the nucleus paragigantocellularis lateralis in the brainstem appear to result in its elimination (Hall *et al*, 1982; Morton *et al*, 1983; Foong & Duggan, 1986).

In addition to receiving these inputs, many of the cells throughout the dorsal horn laminae are projection neurons which relay sensory information to higher areas in the central nervous system. There are several main tracts through which this information may travel.

#### **1.2.4. Ascending Somatosensory Pathways**

**(a) Spinothalamic Tract:** Neurons in the spinal dorsal horn project to thalamic nuclei via the ventrolateral quadrant of the spinal cord, usually contralateral to the side of sensory input (Willis *et al*, 1979). Human studies have shown that lesions in this quadrant may lead to analgesia (Spiller & Martin, 1912), whereas stimulation can result in sensations that are painful (Mayer *et al*, 1975). Tracing experiments based on the retrograde transport of HRP in the rat have demonstrated that neurons primarily in lamina I, III and IV have axons which terminate in the ventro-posterolateral nucleus and the intralaminar complex of the thalamus (Giesler *et al*, 1979; Granum, 1986; Burnstein *et al*, 1990). Antidromic activation in electrophysiological studies has confirmed that neurons of the rat and cat spinothalamic tract (STT) are found in laminae III-V, with many of them being nociceptive, both multireceptive and nocispecific (Dilly *et al*, 1968; Giesler *et al*, 1976).

**(b) Spinoreticular Tract:** Also ascending through the ventrolateral quadrant of the spinal cord, this tract projects to the brainstem reticular formation which in turn relays information to the thalamus (Kevetter & Willis, 1983). HRP labelling provides evidence that laminae VII and VIII cells in the rat ventral horn are the main origin of spinoreticular tract (SRT) units, though some cells in more superficial layers of the dorsal horn are also apparent (Chaouch *et al*, 1983). Menétrey *et al* (1980) found it possible to activate many neurons in laminae III-IV, and a few in laminae I, V and VI, when stimulating the reticular formation. In this study they observed almost half of the cells to be multireceptive and a further fifth were nocispecific.

(c) **Spinomesencephalic Tract:** HRP and fluorescent dye tracing showing prominent projections from laminae I and some in lamina V neurons to the mesencephalic reticular formation and the periaqueductal gray matter (Menétrey *et al*, 1982; Harmann *et al*, 1988; Yeziarski & Mendez, 1991). Electrophysiological evidence from rats also demonstrates that many spinomesencephalic tract (SMT) neurons originate in the marginal zone and respond to noxious cutaneous inputs (Menétrey *et al*, 1980)

(d) **Postsynaptic Dorsal Column System:** Not all axons in the dorsal columns are primary afferent fibres, with an estimated 15% thought to be second order neurons; the postsynaptic dorsal column (PSDC) pathway, some of which relay nociceptive information (Angaut-Petit, 1975). These neurons project to the nucleus gracilis and nucleus cuneatus, with HRP labelling providing evidence that the majority of their cell bodies originate from the laminae II/III border in the rat (Giesler *et al*, 1984). In electrophysiological recording experiments both multireceptive and nocispecific neurons are reported to be among the PSDC destination nuclei (Angaut-Petit, 1975; Brown *et al*, 1983).

As well as these direct routes beyond the spinal cord there are other pathways, including the spinocervical tract (Brown *et al*, 1976; 1977; 1980), which carry information via other spinal cord segments to the brain. There are many neurotransmitters and neuromodulators which may contribute to encoding nociceptive information within the various stages of these pathways, and among them is the ubiquitous excitatory amino acid glutamate.



### 1.3. GLUTAMATE AS A SPINAL NEUROTRANSMITTER

The extensively studied excitatory amino acid (EAA) glutamate has been shown to be a neurotransmitter eliciting fast excitatory responses throughout the central nervous system (Watkins & Evans, 1981; Mayer & Westbrook, 1987). The present study was focused on the role of glutamate (especially by means of metabotropic receptors) in nociceptive processing in the dorsal horn of the spinal cord.

Since glutamate is involved in cellular metabolism, its presence in a neuron cannot immediately indicate neurotransmitter function. However, immunogold electron microscopy studies have extensively demonstrated that throughout the central nervous system there are higher levels of glutamate-like immunoreactivity (Glu-LI) in putative glutamatergic terminals than in any other cellular compartment (Somogyi *et al*, 1986; Montero & Wenthold, 1989; Ottersen 1989a; 1989b; Bramham *et al*, 1990; Broman *et al*, 1990; Maxwell *et al*, 1990a; 1990b; Montero 1990; Zhang *et al*, 1990; Broman & Ottersen, 1992). The presence of Glu-LI at such terminals is also higher around synaptic vesicle clusters (Ji *et al*, 1991; Broman *et al*, 1993). Furthermore, Battaglia & Rustioni (1988) showed with double-labelling immunocytochemistry in DRG cells how the intensity of staining for cytochrome oxidase, an enzyme believed to be a metabolic marker (Wong-Riley, 1979; Wong-Riley & Carroll, 1984), does not correlate with that of glutamate. This would suggest that the cellular complement of this amino acid is not solely for metabolic purposes. Still, when interpreting challenge-induced alterations in cellular glutamate levels, it should be considered that changes in the neurotransmitter pool, since it is much smaller, may be occluded by simultaneous metabolic alterations (Roberts & Hill, 1978).

An attempt to demonstrate the release of glutamate from primary afferent fibres *in vivo* was first made by Roberts (1974). Following electrical stimulation of dorsal column tracts raised glutamate levels were successfully measured in dorsal column nuclei in the brainstem. *In vitro* preparations have since confirmed the presence of glutamate in primary afferent fibres by either measuring changes in its high affinity uptake following dorsal column lesions (Roberts & Hill, 1978) or dorsal root stimulation (Jessel *et al*, 1986; Kawagoe *et al*, 1986). Glutamate was initially inferred to be associated with intrinsic neurons of the dorsal horn by means of biochemical, histochemical and immunocytochemical assays (Rizzoli, 1968; see Fagg & Foster, 1983; Storm-Mathisen & Ottersen, 1987). More recent electron microscopy studies have specifically shown Glu-LI associated with synaptic vesicles



throughout neurons and primary afferent terminals of the superficial dorsal horn (De Biasi & Rustioni, 1988; Miller *et al*, 1988; Maxwell *et al*, 1990a; Broman *et al*, 1993).

For several other reasons glutamate is believed to be involved in the transmission of nociceptive information, in the spinal cord. Glutamate release in the dorsal horn has been demonstrated following noxious stimulation or peripheral inflammation (Skilling *et al*, 1988; Sorkin *et al*, 1992). Ionophoretic application of glutamate was initially shown to cause excitation of spinal neurons by Curtis *et al* in 1959. This and other early studies suggested it played a nonselective excitatory action in the cord (Curtis *et al*, 1960; Ziegelgänsberger & Herz, 1971; Ziegelgänsberger & Puil, 1973). However, more recent evidence indicated that glutamate could be selective for the transmission of nociceptive information. There are higher levels of glutamate in dorsal rather than ventral roots (Graham *et al*, 1967; Duggan & Johnston, 1970) and, more specifically, in dorsal root axons Glu-LI is higher in those which are unmyelinated (Westlund *et al*, 1989). Furthermore, although glutamate is found in DRG cells of all sizes, it is preferentially located in the smaller cells associated with nociception (Duce & Keen, 1983; Salt & Hill, 1983; Cangro *et al*, 1985; Battaglia & Rustioni, 1988). By means of immunocytochemistry, it has also been located in primary afferent terminals fitting the light and electron microscope criteria, in terms of size and morphology, of A $\delta$  and C fibres (De Biasi & Rustioni, 1988). Recording extracellularly from a hamster spinal cord slice preparation, Schneider & Perl (1985; 1988) found that the majority of units in the dorsal horn responding to extracellular glutamate were in the superficial laminae and could be activated by C fibre volleys. Furthermore, the neurons not excited by glutamate were usually only activated by myelinated fibres, and were distributed throughout the remainder of the dorsal horn. These data support a more selective role for this excitatory amino acid. Some studies do however support a further role for glutamate, in mediating low threshold inputs. These will be presented later with specific reference to the receptor subgroups involved.

Intrathecal administration of glutamate produces behavioural hyperalgesia and spontaneous nociceptive behaviour (Aanonsen & Wilcox, 1986; 1987) and increases formalin-induced behavioural responses when given as a pretreatment (Coderre & Melzack, 1992). The subcutaneous injection of formalin into the hindpaw results in an initial acute stage (typically for 0-10 minutes after administration) followed by a prolonged, tonic phase (25-60 minutes). This biphasic effect can be seen both behaviourally as animals lick and bite their paw (Dubuisson & Dennis, 1977; Alreja *et al*, 1984; Seguin *et al*, 1995) and in the

electrophysiological correlate of increased activity of dorsal horn neurons (Dickenson & Sullivan, 1987; Shibata *et al*, 1989). This is therefore used as a model of prolonged noxious stimulation. Sluka & Westlund (1993b) immunohistochemically monitored glutamate levels in the dorsal horn following the induction of arthritis due to an injection of kaolin and carrageenan into the knee joint, and found that its increase correlated with behavioural hyperalgesia.

There are several receptor subtypes through which glutamate (and perhaps other EAA transmitters such as aspartate) may mediate cellular effects. The **ionotropic** group, receptor linked ion channels, consists of (i)  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA/quisqualate, QA), (ii) kainate (KA) and (iii) N-methyl-D-aspartate (NMDA), each named after their preferential agonists. The **metabotropic** glutamate receptors (mGluRs) are coupled through GTP-binding proteins to various second messenger systems (Schoepp *et al*, 1990b).

### **1.3.1. IONOTROPIC GLUTAMATE RECEPTORS**

#### **1.3.1.(a) Non-NMDA Receptors**

Differential effects of AMPA and KA on C-fibre afferents demonstrated that two distinct receptor types existed in the spinal cord (Davies *et al*, 1979). However, since no other agonist or antagonist clearly distinguishes between these receptors they are often regarded collectively, as non-NMDA receptors. Reference is now frequently made to AMPA- and KA-preferring receptors (Partin *et al*, 1993).

Initial expression cloning studies in the *Xenopus* oocyte resulted in the molecular characterisation of two ionotropic glutamate receptor subunits, one each for NMDA (Moriyoshi *et al*, 1991) and for AMPA (Hollmann *et al*, 1989). Following these experiments, novel but homologous sequences were amplified from complementary DNA (cDNA) using the polymerase chain reaction (PCR) with degenerate primers based on the conserved sequence domains. This provided the sixteen ionotropic receptor subunits now being investigated (see Nakanishi *et al*, 1992; Wisden & Seeburg, 1993). *In vitro*, functional AMPA receptors can be composed of one or any two of the GluR1-4 subunits (Boulter *et al*, 1990; Nakanishi *et al*, 1990). It is the GluR2 subunit which provides the receptor with its low divalent cation permeability and linear current-voltage relationship (Jonas & Sakmann, 1992). Kainate-preferring receptors are generated *in vitro* from the subunits GluR5, 6 and 7 (Bettler *et al*, 1990; Egeberg *et al*, 1991; Sommer *et al*, 1992; Bettler *et al*, 1992; Lomeli *et al*, 1992), or KA1 and KA2 (Werner *et al*, 1991; Herb *et al*, 1992; Sakimura *et al*, 1992). The latter two subunits are not functional if expressed alone, but can be combined with GluR5 or 6 to provide viable channels (Herb *et al*, 1992).

#### **(ai) Location in the Spinal Cord**

Ligand binding studies have demonstrated that there are AMPA-preferring receptors throughout the central nervous system (Monaghan *et al*, 1984) and in the spinal cord [ $^3\text{H}$ ]AMPA binds preferentially in the substantia gelatinosa of the dorsal horn (Albin *et al*, 1993; Henley *et al*, 1993). Subunits thought to be primarily involved in the AMPA-preferring receptor complex (GluR1-4) have been shown by means of *in situ* hybridisation histochemistry (ISHH) and immunocytochemistry to be present throughout the dorsal horn with GluR2 showing strongest expression throughout, and being concentrated in the superficial laminae (Furuyama *et al*, 1993; Sato *et al*, 1993; Tölle *et al*, 1993). Similarly, [ $^3\text{H}$ ] kainic acid binding shows kainate receptor sites to be widespread throughout the whole of

the central nervous system, including the substantia gelatinosa (Monaghan & Cotman, 1982), and messenger RNA (mRNA) for KA2, a kainate receptor subunit, is abundant in the dorsal horn (Tölle *et al*, 1993). Also, functional KA receptors have been characterised in DRG cells (Huettnner, 1990).

**(aii) Electrophysiological Evidence for a Role in Sensory Processing**

Evidence from studies investigating the latency of different synaptic transmission components suggests that AMPA/QA and KA receptors are involved in monosynaptic pathways in the spinal cord and they are thus believed to mediate the fast synaptic transmission brought about by glutamate release (Jahr & Jessell, 1985; Jahr & Yoshioka, 1986; Gerber & Randic, 1989a), whereas NMDA receptors are thought to contribute towards prolonged changes in excitability and polysynaptic inputs (Davies & Watkins, 1983; Davies & Lodge, 1983; Peet *et al*, 1983; Schneider & Perl, 1985; Schouenborg & Sjolund, 1986; King *et al*, 1988; Gerber & Randic, 1989a; Morris, 1989).

Electrically-evoked C fibre inputs can be decreased by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; an antagonist at the non-NMDA type ionotropic EAA receptors, Blake *et al*, 1988; Alford & Grillner, 1990), to a greater extent than A $\delta$  fibre inputs (Yoshimura & Jessell, 1990). AMPA has been shown to increase the response of spinal neurons to noxious mechanical stimulation (Aanonsen *et al*, 1990), whereas CNQX can decrease dorsal horn neuron responses to both innocuous and noxious mechanical stimuli (Dougherty *et al*, 1992, Neugebauer *et al*, 1993a), noxious thermal and A $\beta$  and A $\delta$  fibre strength electrical stimuli (Dougherty *et al*, 1992). Paleckova *et al* (1992) demonstrated that C-fibre strength electrical stimulation of the sciatic nerve evoked the release of glutamate which could be blocked by CNQX, suggesting this was via AMPA receptors on interneurons. CNQX can prevent the development of arthritis-induced hyperexcitability (Neugebauer *et al*, 1993b) as well as reducing the response of dorsal horn neurons to innocuous and noxious mechanical stimulation in the inflamed state (Neugebauer *et al*, 1994a). Radhakrishnan & Henry (1993) found that CNQX blocked innocuous hair movement responses only, leaving thermal nociceptive responses completely unaffected. Haley *et al* (1990) showed that a non-selective EAA antagonist ( $\gamma$ -D-glutamylglycine, DGG) could decrease both early and late phases of the formalin-induced firing of dorsal horn neurons, yet an NMDA antagonist inhibited only the second phase, thus demonstrating a role for non-NMDA receptors, but not NMDA receptors, in the acute phase of the formalin response.

**(aiii) Behavioural Evidence for a Role in Sensory Processing**

The role of non-NMDA receptors in behavioural analgesia is somewhat controversial. Behavioural testing has demonstrated how intrathecally administered AMPA or kainate results in caudally-directed biting and scratching (Aanonsen & Wilcox, 1986; Kitto *et al*, 1992). Malmberg & Yaksh (1992a) found that intrathecally administered AMPA resulted in thermal hyperalgesia in the paw, accompanied by acute agitation of the rats. Both effects were reversed by CNQX. In contrast Kitto *et al* (1992) did not observe such decreases in withdrawal latencies to noxious thermal stimulation. Intrathecal CNQX has been shown to have antinociceptive effects in the hot plate test, formalin-induced irritation of the hindlimb (Näsström *et al*, 1992), carrageenan-induced hyperalgesia (Ren *et al*, 1992) and, when given as a pre-treatment, can decrease thermal hyperalgesia that was elicited by chronic constrictive injury (CCI) of the sciatic nerve (Mao *et al*, 1992). The release of glutamate due to kaolin/carrageenan-induced arthritis is also decreased by CNQX (Sluka & Westlund, 1993a). Furthermore, a second AMPA antagonist, 2,3-dihydroxy-6-nitro-sulphamoyl-benzo(f)quinoxaline (NBQX) blocks the behavioural hypersensitivity produced by laser-induced spinal ischemia (brought about by an intravascular photochemical reaction, involving the activation of systemically injected Erythrosin B by an argon laser beam, Xu *et al*, 1993). In contrast,Coderre & Melzack (1992) found that AMPA did not increase, and CNQX did not decrease, the nociceptive behaviour induced by formalin. Non-NMDA ionotropic receptors clearly appear to play some role in the transmission of nociceptive information, however the exact physiological significance of these receptors in sensory inputs to the spinal cord is still unresolved.

### **1.3.1.(b) NMDA Receptors**

NMDA receptors have been located throughout the brain and central nervous system (Greenamayre *et al*, 1984; Monaghan & Cotman, 1985; Moriyoshi *et al*, 1991). There is compelling evidence that they play a role in excitability changes such as long-term potentiation (LTP) in the hippocampus (Collingridge *et al*, 1983; Herron *et al*, 1986; Morris *et al*, 1986; Bliss & Lynch, 1988). Under normal physiological conditions the channel which is linked to this receptor is blocked by magnesium ( $Mg^{++}$ , Mayer *et al*, 1984; Nowak *et al*, 1984) and the receptor is unlikely to exert any substantial cellular effects in such an environment. However, this  $Mg^{++}$  block may be alleviated by partial depolarisation of the membrane (Ben-Ari *et al*, 1992), perhaps by another neurotransmitter/modulator. The absence of glycine in *in vitro* media led to the discovery that the NMDA receptor has a glycine-sensitive site which must be occupied for functional activity (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988). Blockade by NMDA receptor antagonist 2-amino-5-phosphonic acid (APV) of the slow component of neuronal activity, evoked by electrical stimulation or agonist ionophoresis, has led to the theory that NMDA receptors mediate the slow synaptic responses brought about by glutamate (Dale & Roberts, 1985; Dale & Grillner, 1986; Collingridge *et al*, 1988; King *et al*, 1988).

As described in section 1.3.2.1. for non-NMDA receptors, the subunits of the NMDA receptor have been cloned and expressed *in vitro*. This receptor is a dimeric structure which can be composed of the NR1 subunit and any one of four NR2 (A-D) units (Monyer *et al*, 1992; Kutsuwada *et al*, 1992; Meguro *et al*, 1992; Ikeda *et al*, 1992; Ishii *et al*, 1993). The properties of the receptor, including the strength of  $Mg^{++}$  block and the sensitivity to glycine, are highly dependent on the NR2 subunit utilised (Monyer *et al*, 1992; Meguro *et al*, 1992; Ishii *et al*, 1993). The NR1 unit is expressed ubiquitously in the brain and spinal cord (Moriyoshi *et al*, 1991) and it is the NR2 subunits which show temporal and spatial specifications (e.g. in the spinal cord see Tölle *et al*, 1993).

#### **(bi) Location in the Spinal Cord**

There are various lines of evidence indicating the presence of NMDA receptors in the spinal cord. Ligand binding (Greenamayre *et al*, 1984; Monaghan & Cotman, 1985; Albin *et al*, 1993; Henley *et al*, 1993) indicates that they are present in the superficial dorsal horn, though Nagy *et al* (1994) suggest that this may be due to receptors on the dendrites of deeper cells. The mRNA for NR1, a glutamate-binding subunit of the NMDA receptor (Kumar *et al*, 1991), has been located in the



dorsal horn. Furuyama *et al* (1993) observed diffuse and low intensity hybridisation throughout, but strongest in the deeper layers. Tölle *et al* (1993) reported an abundant, widespread and even expression of NR1 mRNA over all of the grey matter, including the substantia gelatinosa, along with low levels of NR2C and D. The mRNA for NR1 has also been found in dorsal root ganglia (Shigemoto, 1992).

**(bii) Electrophysiological Evidence for a Role in Sensory Processing**

Spinal neurons can be excited by NMDA (Anis *et al*, 1983; King *et al*, 1988; Sher & Mitchell, 1990a; Dougherty & Willis, 1991b), in some cases those receiving nociceptive inputs respond preferentially (Aanonsen *et al*, 1990). Under normal physiological conditions, i.e. without any deliberate damage or injury, NMDA receptors do appear to have a role to play, although less clearly than the AMPA receptor. Responses to noxious mechanical and thermal stimulation of the skin can be enhanced by NMDA (Aanonsen *et al*, 1990) and one study found an increase in the response to innocuous brush (Dougherty & Willis, 1991a). In accord, under normal conditions NMDA receptor antagonist APV has also been shown to reduce neuronal responses to noxious mechanical, thermal and electrical stimulation (Dougherty *et al*, 1992; Radhakrishnan & Henry, 1993; Raigorodsky & Urca, 1990), as well as noxious pressure to the knee joint (Neugebauer *et al*, 1993a). Conversely, Headley *et al* (1987) found that ketamine, a dissociative anaesthetic and NMDA antagonist, failed to alter any sensory responses of dorsal horn neurons under "normal" conditions. Davies & Lodge (1987) also found that this compound did not produce any consistent effect until wind-up (a sensitisation phenomenon, see 1.3.1.(biii)) was induced. These two studies may differ from the others simply because of the drugs used.

A role for NMDA receptors in the induction and maintenance of a pathophysiological or sensitised state is strongly indicated. In dorsal horn neurons, the NMDA receptor-selective antagonists, APV and ketamine can block hindlimb-ischaemia-evoked firing (Sher & Mitchell, 1990b) and 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-10-imine maleate (MK-801) reduces background activity and the response to noxious heating of the skin, following complete Freund's adjuvant (CFA)-induced inflammation (Ren *et al*, 1992b). Intravenously or ionophoretically administered NMDA antagonists can decrease neuronal responses to innocuous and noxious stimulation of the inflamed knee (Schaible *et al*, 1991; Neugebauer *et al*, 1994a) and prevent the development of neuronal hyperexcitability induced by acute arthritis (Neugebauer *et al*, 1993b).

The second peak of firing evoked in dorsal horn neurons by subcutaneous formalin (see p15 for details of the model) can be markedly reduced if NMDA receptor antagonists are given as a pretreatment or administered during activity, whereas the first acute phase is largely unaffected (Haley *et al*, 1990). Similarly, Woolf & Thompson (1991) found that reflex facilitation induced by either electrical stimulation or the cutaneous application of mustard oil, a chemical irritant which selectively activates C-fibres (Heapy *et al*, 1987; see section 3.2. for details of this model), could be blocked by NMDA receptor antagonists if they were given before or after the induction of such sensitisation. Both of these experimental paradigms are thought to be analogous to the phenomenon of "wind up", an event which may occur as part of central sensitisation.

**(biii) Wind Up and Central Sensitisation**

Mendell (1966) initially described "wind up" as the increased firing of a neuron due to repetitive electrical stimulation of C afferent fibres. The discharge of a cell could be increased if the stimulus was applied at greater than or equal to one impulse every two or three seconds ( $\geq 0.3\text{Hz}$ ). Wind up is therefore thought to be frequency dependent and relies only on the features of the afferent (C-fibre) input, and not the post-synaptic cell. A central origin for wind up has been proposed, as it occurs without any rise in the size of the incoming volley (Davies & Lodge, 1987). This phenomenon typically consists of a transient early phase, thought to be A-fibre mediated, and a longer-lasting second phase evoked by C-fibre activity (Davies & Lodge, 1987). However, Headley & Grillner (1990) suggested three components of wind up. The initial one being NMDA-insensitive, followed by an NMDA sensitive event and finally a third, longer lasting episode likely to be mediated by some other neurotransmitter (e.g. tachykinins, see Thompson *et al*, 1993).

Studies using the electrically-evoked wind up model have consistently observed the ability of NMDA receptor antagonists to decrease or abolish ongoing C fibre activity (Davies & Lodge, 1987; Dickenson & Sullivan, 1987; Dickenson & Sullivan, 1990; Thompson *et al*, 1990), thus implicating the NMDA receptor in prolonged nociceptive inputs. The non-selective EAA receptor antagonist kynurenate can decrease the two phases of wind up suggested by Davies & Lodge (1987), whereas ketamine, a selective NMDA antagonist, only the second (Davies & Lodge, 1987). Haley *et al* (1990) observed a similar outcome in the formalin model



using DGG and APV. These studies, along with further electrophysiological experiments in which electrically-evoked synaptic responses have been broken down into separate components (Gerber & Randic 1989a; 1989b; Dickenson & Sullivan, 1990), have therefore suggested that non-NMDA receptors are involved in both low and high threshold inputs to the dorsal horn, whereas NMDA receptors play a more selective role in high threshold, particularly C-fibre, inputs.

The activity-dependent alterations in dorsal horn neuron responsiveness brought about during wind-up are thought to be part of the phenomenon of central sensitisation. This process is initiated by prolonged noxious or inflammatory stimulation of these neurons, with a normally innocuous A $\beta$  fibre input resulting in an exaggerated, painful response ("allodynia"; Woolf & King, 1990). Further consequences of central sensitisation can be seen as an elevated level of neuronal firing in response to mechanical (Kenshalo *et al*, 1979; 1982; McMahon & Wall, 1984; Hylden *et al*, 1989; Laird & Cervero, 1989; Simone *et al*, 1991; Woolf & King, 1990), thermal (Kenshalo *et al*, 1979; Simone *et al*, 1991) and chemical (Simone *et al*, 1991; Dougherty & Willis, 1992) stimulation, as well as a facilitation of nociceptive flexor reflexes (Woolf & Wall, 1986). An expansion of the cutaneous receptive field is also observed (McMahon & Wall, 1984; Cervero *et al*, 1990; Woolf & King, 1990; Treede *et al*, 1992). An additional manifestation of central sensitisation is secondary hyperalgesia, whereby an area outwith the site of injury (often well beyond the region of neurogenic inflammation, perhaps even a contralateral limb) experiences increased sensitivity to noxious stimulation - changes which cannot be fully explained by peripheral mechanisms. Evidence for a central component includes a demonstration by LaMotte *et al* (1991) that local anaesthetic in the primary area prevents capsaicin-induced secondary hyperalgesia to mechanical stimuli only if given before the capsaicin injection, but not after. Also, in behavioural studies, secondary hyperalgesia, due to noxious thermal injury of the ipsilateral paw fifteen minutes previously, persists in an uninjured contralateral paw (Coderre & Melzack, 1991). Pretreatment with intrathecal APV prevented the development of this hyperalgesia.

**(biv) Behavioural Evidence for a Role in Sensory Processing**

The involvement of NMDA receptors in nociception has been demonstrated in a variety of behavioural tests, including those mentioned above as reflecting at least in part, secondary hyperalgesia. Intrathecal NMDA itself can induce biting and scratching behaviour indicative of a painful state (Aanonsen & Wilcox, 1986; 1987; Raigorodsky & Urca, 1987; Coderre & Melzack, 1991) and this can be reversed by the NMDA receptor antagonist APV (Aanonsen & Wilcox, 1986; Raigorodsky & Urca, 1990). Also, when delivered as a pretreatment NMDA can potentiate similar nociceptive behaviour observed in response to a subcutaneous injection of formalin (Coderre & Melzack, 1992). Kolhekar *et al* (1993) found that a higher dose of NMDA could give rise to reflex facilitation in the thermal tail flick test, with a lower dose inducing hypoalgesia. Spinalisation, by local cold blocking of the cord, resulted in abolition of the NMDA-induced inhibition, but no change in the facilitation of the reflex. It was therefore suggested that a local mechanism was employed for NMDA-evoked hyperalgesia but the involvement of supraspinal sites was required in NMDA-evoked hypoalgesia. Further support for a local mechanism being responsible for this NMDA facilitation is that it can also be observed in the hemisectioned spinal cord *in vitro* (Thompson *et al*, 1992; Rusin *et al*, 1993b) and in skin-hindlimb (Thompson *et al*, 1990) preparations, where supraspinal circuitry is irrelevant.

Antagonists to NMDA receptors have been shown to be antinociceptive in a number of behavioural studies involving sensitisation (and on some occasions in models not considered to involve sensitisation). In the acute state, latencies in the thermal tail flick reflex (Cahusac *et al*, 1984; Raigorodsky & Urca, 1990) and the hot plate response (Cahusac *et al*, 1984; Näsström *et al*, 1992) are both delayed by NMDA antagonists (APV, 2-amino-7-phosphonoheptanoic acid (AP7) and 3-(2-carboxypiperazine-4-yl)propylphosphonate (CPP)). The latencies of paw withdrawal to noxious mechanical stimulation is also prolonged by APV administration (Cahusac *et al*, 1984). In more chronic situations NMDA receptor antagonists are also analgesic: thermal hyperalgesia induced by carrageenan is reduced by MK-801, APV & CPP, Ren *et al*, 1992a; 1992b); sciatic nerve ligation hyperalgesia is reduced by MK-801 (Davar *et al*, 1991; Yamamoto & Yaksh, 1992a; 1992b); and by 1-hydroxy-3-aminopyrrolidone-2 (HA 966, Mao *et al*, 1992) and heat injury hyperalgesia is reduced by APV (Coderre & Melzack, 1991). Similarly, autotomy evoked by sciatic nerve ligation is prevented by intrathecally applied MK-801 or APV (Seltzer *et al*, 1991). Licking of the paw induced by the subcutaneous

administration of formalin can likewise be decreased by APV, MK-801, AP7 & CPP; (Murray *et al*, 1991;Coderre & Melzack, 1992; Näsström *et al*, 1992; Vaccarino *et al*, 1993), as can the mechanical hyperalgesia resulting from intraplantar injection of complete Freund's adjuvant (Ren *et al*, 1992b; Ren & Dubner, 1993).

It would therefore appear that NMDA receptors are involved in the transmission of nociceptive information, particularly after the system has undergone some form of sensitisation. They mediate high threshold inputs via A $\delta$  and C fibres. Non-NMDA receptors, on the other hand, have a more general function, in that they do play some role in nociception, but are also involved in low threshold A $\beta$  fibre inputs. There is a further group of receptors by which glutamate may exert its effects, that is the metabotropic group, which, rather than acting via ion channels, are directly coupled to intracellular signalling pathways.

### 1.3.2. METABOTROPIC GLUTAMATE RECEPTORS

A metabotropic type of glutamate receptor was initially implicated following the enhancement of inositol phosphate hydrolysis by glutamate, in mammalian brain cells (Sladeczek *et al*, 1985; Nicoletti *et al*, 1986). Sugiyama *et al* (1987) later demonstrated a quisqualate (QA)-preferring receptor directly activating inositol phosphate metabolism, via a G protein, which could not be blocked by Joro spider toxin, a known blocker of ionotropic QA receptors.

#### (a) Cloning and Expression of Receptor Subtypes

Masu *et al* (1991) were the first to isolate a metabotropic glutamate receptor (mGluR) cDNA, now known as mGluR1a. Using the mRNA of mGluR1a as a probe for sequences with high homology, cloning and expression of the other receptors quickly followed, with a total of 8 to date (Tanabe *et al*, 1992; Abe *et al*, 1992; Nakajima *et al*, 1993; Okamoto *et al*, 1994; Duvoisin *et al*, 1995). Three further splice variants of mGluR1 have been found: mGluR1b (Tanabe *et al*, 1992) and mGluR1c (Pin *et al*, 1992), both of which are around 300 amino acids shorter than mGluR1a. More recently a truncated (presumably non-functional) sequence termed mGluR1e has also been detected (Pin & Duvoisin, 1995). Similarly, a splice variant of mGluR4 has been cloned, resulting in an mGluR4b protein which is 127 residues longer than the original (Simoncini *et al*, 1993). Finally, there is an mGluR5b variant which is 32 amino acids longer than mGluR5a (Minakami *et al*, 1993). It is not yet clear whether the splice variants have different functional roles or not.

Most of the receptor subtypes are expressed throughout the central nervous system, however, some have specific regions of preference: mGluR1 mRNA is highly expressed in the hippocampus and cerebellar neurons (Masu *et al*, 1991) whereas mGluR2 is found primarily in Golgi cells of the cerebellum and the olfactory bulb (Tanabe *et al*, 1992; Ohishi *et al*, 1993). mGluR3 is prominently expressed in the cerebral cortex and dentate gyrus, but is also very abundant in glial cells (Tanabe *et al*, 1992; 1993). Granule cells of the cerebellum appear to be rich in mRNA for mGluR4 (Tanabe *et al*, 1992), as are the cerebellum, thalamus and olfactory bulb (Tanabe *et al*, 1993), while mGluR5 has been localised in the pyramidal cells in areas CA1-CA4 of the hippocampus and dentate gyrus granule cells (Abe *et al*, 1992). Messenger RNA for mGluR6 was found exclusively in the retina, with no expression elsewhere in the brain (Nakajima *et al*, 1993), while a

**Table 1.1.**

**A summary of the subgroups of metabotropic glutamate receptors**

Metabotropic glutamate receptors have been divided into three distinct groups, on the basis of their signal transduction pathways and agonist specificity.

**IP<sub>3</sub>**: Inositol-1-4-5-triphosphate; **Ca<sup>++</sup>**: calcium; **cAMP**: cyclic AMP;  
**QA**: quisqualate; **Glu**: glutamate; **ACPD**: (1S,3R)-1-aminocyclopentane-1-3-dicarboxylic acid; **L-AP4**: L-aminophosphonobutanoate

SUBGROUP	RECEPTOR	SIGNAL TRANSDUCTION	AGONIST SELECTIVITY
I	1	IP <sub>3</sub> / Ca <sup>++</sup>	QA>glu≥ibotenate>ACPD
	5		
II	2	inhibition of cAMP	glu≥ACPD>ibotenate>>QA
	3		
III	4	inhibition of cAMP	L-AP4>glu>>ACPD
	6		
	7 (and 8)		

widespread distribution of mGluR7 mRNA was observed (Okamoto *et al*, 1994). Finally, mGluR8 has been primarily located in the olfactory bulb and weakly in the retina (Duvoisin *et al*, 1995). Although several mGlu receptor mRNAs have been located in the spinal cord, none of them were found to be prominently expressed.

**(b) Intracellular Signalling Pathways & Receptor Subgroups**

All metabotropic glutamate receptors are, by definition, coupled to GTP-binding proteins. They appear to elicit not only inositol phosphate hydrolysis as originally described (Nicolletti *et al*, 1986; Challis *et al*, 1988; Sladeczek *et al* 1988; Baird *et al*, 1991) but many other intracellular changes. These receptors can activate protein kinase C (PKC), via diacylglycerol (Manzoni *et al*, 1990; Behnisch & Reymann, 1993), evoke the release of arachidonic acid (Aramori & Nakanishi, 1992), increase (Aramori & Nakanishi, 1992; Winder & Conn, 1992; 1993), decrease (Manzoni, *et al*, 1990; Cartmell *et al*, 1992; Schoepp & Johnson, 1992) cyclic AMP (cAMP) formation, elevate intracellular calcium (Murphy & Miller, 1988; 1989) or activate phospholipase D (Boss & Conn, 1992). Downstream modulation of various ion channels is also evident (Lester & Jahr, 1990; Fagni *et al*, 1991; Bleakman *et al*, 1992; Cerne & Randic, 1992). Due to the complexity of cellular activity, these initial steps induced by the binding of glutamate to a metabotropic receptor may then go on to activate, or indeed inhibit, further intracellular pathways.

The eight different mGluRs known at present (Nakanishi, 1992; Okamoto *et al*, 1994; Duvoisin *et al*, 1995) have been divided into three groups based on their mRNA sequence homology, coupling to intracellular signalling pathways and their agonist (and more recently antagonist) pharmacology:

(I) *mGluRs 1 and 5*: These receptors are directly coupled to inositol phosphate hydrolysis/calcium signal transduction (see Figure 1.3.(a)) and are most sensitive to quisqualate (Houamed *et al*, 1991; Masu *et al*, 1991; Abe *et al*, 1992). A relatively new compound, 3,5-dihydroxyphenylglycine (3,5-DHPG), has also been shown to have preferential agonist properties for these receptors (Schoepp *et al*, 1994). (S)-4-carboxy-3-hydroxyphenylglycine ((S)-4C3HPG and (S)-CHPG) are antagonists at Group I mGluRs, but it is possible that their potency may be species-dependent (Joly *et al* 1995; Kingston *et al*, 1995). Cyclothiazide (CTZ), a selective mGluR1 antagonist, can pharmacologically distinguish between these two receptors (Sharp *et al*, 1994).



(II) *mGluRs 2 and 3*: The receptors in this group are negatively linked to the formation of cAMP (see Figure 1.3.(b)) and are highly sensitive to (1S,3R)-1-aminocyclopentane-1-3-dicarboxylic acid ((1S,3R)-ACPD; Nakanishi, 1992; Tanabe *et al*, 1992; 1993) as well as (2S,3S,4S)- $\alpha$ -(carboxycyclopropyl)glycine (CCG-I; Hayishi *et al*, 1992) and (2S,1'R,2'R,3'R)-2-(dicarboxycyclopropyl)glycine (DCG-IV; Hayishi *et al*, 1993).  $\alpha$ -methyl-L-CCG-I (MCCG) has been shown to selectively antagonise responses induced by CCG-I, and not (1S,3R)-ACPD, in neonatal rat primary afferent terminals (Jane *et al*, 1994).

(III) *mGluRs 4, 6 and 7 (and 8)*: Finally, the third group are also involved in an inhibition of cAMP production (see Figure 1.3.(b)). These receptors are insensitive to (1S,3R)-ACPD, but are easily activated by L-aminophosphonobutanoate (L-AP4, Thomsen *et al*, 1992; Nakajima *et al*, 1993; Tanabe *et al*, 1993; Okamoto *et al*, 1994; Duvoisin *et al*, 1995). Specific antagonism of L-AP4-, and not (1S,3R)-ACPD-, induced excitations in primary afferent terminals has been demonstrated using  $\alpha$ -methyl-L-AP4 (Jane *et al*, 1994).

(See table 1.1. for summary).

(c) **Electrophysiological Evidence for a Role in Sensory Processing**

There have been many studies investigating the pharmacological profiles of the mGluR subtypes, primarily using phenylglycine derivatives from Watkins and his colleagues (see Watkins & Collingridge, 1994 for review). These experiments, mainly *in vitro*, have demonstrated the existence of functional mGluRs in many areas of the brain including the somatosensory cortex (Cahusac, 1994), visual cortex (Kato, 1993), olfactory cortex (Collins, 1993), olfactory bulb (Hayashi *et al*, 1993), nucleus of the tractus solitarius (Glaum *et al*, 1993), striatum (Lombardi *et al*, 1993), thalamus (Birse *et al*, 1993; Jane *et al*, 1993; Eaton *et al*, 1993a; 1993b; Salt & Eaton 1995a; 1995b) and the hippocampus (Harvey *et al*, 1991; Otani & Ben-Ari, 1991; Bortolotto & Collingridge, 1992; 1993; Bashir *et al*, 1993; Behnisch & Reymann, 1993). However, the number of physiological studies concerning nociception or the spinal cord have been relatively few. Several *in vivo* investigations into the role of mGluRs in somatosensory processing in the thalamus have been made. Excitatory neuronal responses evoked in thalamic neurons by noxious thermal, but not innocuous air jet, stimulation can be selectively inhibited by mGluR antagonists (S)-CHPG, (S)-carboxyphenylglycine ((S)-4CPG) and  $\alpha$ -methyl-4-carboxyphenylglycine (MCPG or  $\alpha$ -MeCPG; Eaton *et al*, 1993a; 1993b). Later, using selective compounds, Salt & Eaton (1995a; 1995b) showed how Group II and III mGluRs may be presynaptically located on GABAergic terminals in the ventrobasal (VB) thalamus, and play a role in the disinhibition of air jet- and electrically-evoked somatosensory inputs. From their studies they also suggest a postsynaptic mGluR1, as VB neurons are excited by both (1S,3R)-ACPD and 3,5-DHPG.

Regarding the spinal cord, *in vitro* studies have shown mGluR agonist (1S,3R)-ACPD can potentiate the responses induced by ionotropic receptor agonists in both isolated dorsal horn neurons and in a spinal cord slice preparation (Bleakman *et al*, 1992; Cerne & Randic, 1992). A recent *in vivo* study confirming this potentiation used more specific agonists and suggested the action is through either Group I or II mGluRs (Bond & Lodge, 1995). Jones & Headley (1995) found a similar rise in ionotropic agonist-evoked activity, however taking increases in background activity into account they concluded the 'potentiation' was not a specific interaction between ionotropic and metabotropic glutamate receptors. Neugebauer *et al*, (1994b) found mGluR antagonist L-1-amino-3-phosphonopropionic acid (L-AP3) reduced the response to pressure applied to the knee following the induction of inflammation, but not before. In contrast to this, in primate STT neurons Palecek *et al* (1994) found (1S,3R)-ACPD, at a low, but not high, dose enhanced brief neuronal responses to innocuous brushing of the receptive field. They did not observe either dose to enhance the neuronal response to brief noxious stimulation. Also, Thompson *et al* (1992) found no effect of L-AP3 on C-fibre-evoked ventral root potentials in a neonatal *in vitro* spinal cord preparation. However, this could have been due to the age of the animal, as the potency of L-AP3 seems to depend on it (Schoepp & Johnson, 1989a; 1989b), and the developmental stage of the fibres involved.

It would appear from the data available at present that mGluRs may play a role in prolonged nociception or sensitisation, but perhaps less so in brief somatosensory inputs, at least in the spinal cord. Another synaptic event which is thought to be analogous to sensitisation, long-term potentiation, has been the subject of much investigation with respect to mGluRs.

#### **(d) Long-Term Potentiation Studies**

Until recently long-term potentiation (LTP), the activity-dependent phenomenon whereby a long lasting efficacy in synaptic transmission is brought about following a brief period of high frequency axonal stimulation, has been mainly attributed to NMDA receptors (for review see Collingridge, 1992). However, mGluRs are also known to play a role in this event - analogous to the sensitisation observed in dorsal horn neurons during sustained nociceptive inputs. Initial studies showed that (1S,3R)-ACPD could induce (Bortolotto & Collingridge, 1992; Bashir *et al*, 1993), mediate (Otani & Ben-Ari, 1991) or augment (McGuinness *et al*, 1991; Anikszten *et al*, 1992) LTP in the hippocampus. Later Bortolotto & Collingridge

(1993) demonstrated that an ionotropic receptor-independent LTP could be evoked using (1S,3R)-ACPD, without the accompanying short term potentiation component normally seen with NMDA-induced LTP. No tetanic stimulation was necessary for this to occur, although pathways from CA1-CA3 had to remain intact. Antagonist studies using L-AP3 (Behnisch *et al*, 1991; Izumi *et al*, 1991; Behnisch & Reymann, 1993) and (RS)- $\alpha$ MeCPG (Bashir *et al*, 1993; Richter-Levin *et al*, 1994; Riedel *et al*, 1995) have illustrated how LTP is sensitive to mGluR inactivation - particularly during, or immediately after the induction of, tetanization (Behnisch & Reymann, 1993). Behaving rats also show a sensitivity to  $\alpha$ MeCPG, as they demonstrate a disrupted performance in spatial learning (water maze) following intraventricular administration of this compound (Richter-Levin *et al*, 1994).

#### **(e) Behavioural Evidence for a Role in Sensory Processing**

There have been few behavioural experiments concerning mGluRs with regard to long-term potentiation or nociception. Coderre & Melzack (1992) found that the behavioural response to formalin was enhanced if mGluR agonist (1S,3R)-ACPD was given intrathecally as a pretreatment. A similar augmentation in behaviour brought about by NMDA administration could be increased even further if the two compounds were given together. However, they did not observe any analgesic effect of L-AP3 in the same, or later (Coderre & Empel, 1994) experiments.

Metabotropic glutamate receptors therefore appear to be involved in synaptic events in a variety of areas in the central nervous system. They undoubtedly have a role to play in the transmission of nociceptive information, and this role may be more substantial in a sensitised state. However mGluRs are only one of a number of contributors to this kind of role, since several other receptors have been implicated in nociception and sensitisation, including those which are activated by the tachykinins.

### **1.4. TACHYKININS**

The mammalian tachykinins are a family of neuropeptides characterised by their common carboxy terminal sequence Phe-X-Gly-Leu-Met. The family includes substance P (SP), neurokinin A (NKA), neurokinin B (NKB), neuropeptide K (NPK)

and neuropeptide  $\gamma$  (NP $\gamma$ ). There are two preprotachykinin (PPT) genes responsible for tachykinin production. Preprotachykinin I (PPT I), consists of three variants,  $\alpha$ -,  $\beta$ - and  $\gamma$ -PPT, the first of which encodes SP only, whereas  $\beta$ - and  $\gamma$ -PPT result in both SP and NKA mRNA expression, accompanied by NPK and NP $\gamma$  respectively (Nawa *et al*, 1984; Krause *et al*, 1987). The second gene, PPT II is responsible for NKB production (Bonner *et al*, 1987). Expression of  $\alpha$ -PPT I mRNA in the central nervous system accounts on average for less than 0.5% of the total level of PPT I ( $\beta$ -PPT and  $\gamma$ -PPT are 22% and 78% respectively; Carter & Krause, 1990), it therefore seems likely that SP mRNA is rarely expressed, or SP released, alone. In further support of this, immunohistochemical analysis shows how nearly all SP-LI-labelled DRG cells are also positive for NKA-LI (Dalsgaard *et al*, 1985).

There are three receptors through which the tachykinins exert their effects: NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub>, at which SP, NKA and NKB are the respective preferential endogenous ligands (Regoli *et al*, 1987; Maggi *et al*, 1993). There is evidence that pharmacological subtypes of NK<sub>1</sub> and NK<sub>2</sub> receptors exist (especially between groups of species, see Maggi *et al*, 1993, for review). All three receptors are linked via G-proteins to phosphoinositide metabolism (Watson & Downes, 1983; Bristow *et al*, 1987; Guard *et al*, 1988; Nakajima *et al*, 1992). The present study concerns the role of NK<sub>1</sub> and NK<sub>2</sub> receptors in sensory processing.

#### **1.4.1. Location in the Spinal Cord**

##### **(a) Substance P and NK<sub>1</sub> Receptors**

Using radioimmunoassay, immunohisto- and immunocyto- chemical techniques, the presence of SP and SP-LI has been detected in the dorsal horn, predominantly in the superficial layers, and dorsal root fibres (Hökfelt *et al*, 1975; 1977; Gibson *et al*, 1981; Charnay *et al*, 1983; Ogawa *et al*, 1985). The SP of primary afferent origin has been shown to come particularly from small unmyelinated (C) fibres (Hökfelt *et al*, 1980). Substance P has also been located in intrinsic neurons and some descending fibres (Jessell *et al*, 1979; Ogawa *et al*, 1985; Yaksh *et al*, 1988), as demonstrated by nerve section or dorsal rhizotomy. *In situ* hybridisation histochemistry has detected mRNA for SP (probing for a sequence common to  $\alpha$ -,  $\beta$ - and  $\gamma$ -PPT) throughout the central nervous system (Haran *et al*, 1989), including high expression in laminae I and II of the dorsal horn (Warden *et al*, 1988). Although ligand binding studies have revealed NK<sub>1</sub> receptors predominantly, but not

exclusively in laminae I, II and X (Yashpal *et al*, 1990), mRNA for the receptor is not highly abundant in these areas (Maeno *et al*, 1993). Studies utilising newly available antibodies directed against the NK<sub>1</sub> receptor have revealed a distribution similar to that observed in ligand binding studies (Bret-Dibat *et al*, 1994; Brown *et al*, 1995).

#### **(b) Neurokinin A and NK<sub>2</sub> Receptors**

NKA, like SP, is synthesised in primary sensory neurons (Harmar & Keen, 1984) and found in fine afferent fibres (Kimura *et al*, 1983; Sundler *et al*, 1983; Ogawa *et al* 1985). Indeed, Ogawa *et al* (1985) showed it has a similar distribution to SP throughout dorsal roots, DRG cells and the dorsal horn. The preferred receptors for NKA (NK<sub>2</sub>) have been shown by radioligand binding to be limited to the superficial laminae of the dorsal horn, unlike NK<sub>1</sub> receptors which are present in both sensory and motor systems (Yashpal *et al*, 1990). Using antagonists which have varying activity at putative NK<sub>2</sub> receptor subtypes Wiesenfeld-Hallin *et al*, (1994) have suggested the existence of at least two subtypes in the rat spinal cord, suggesting that NK<sub>2A</sub> is the one likely to mediate NKA-induced excitation. Attempts have been made to locate the mRNA for NK<sub>2</sub> receptors in the nervous system, but levels in spinal cord have so far proved too low to quantify (Poosch *et al*, 1991; Takeda & Krause, 1991; R.M.C. Parker, PhD thesis, 1994).

### **1.4.2. Evidence for a Role of NK<sub>1</sub> Receptors in Sensory Processing**

#### **(a) Electrophysiological Studies**

Upon ionophoretic application, SP can excite dorsal horn neurons (Henry, 1976; Zieglgänsberger & Tulloch, 1979), resulting in a slow postsynaptic depolarization (Urban & Randic, 1984; Randic *et al*, 1988), and in laminae I - II it selectively activates those neurons responsive to noxious stimuli (Randic & Miletic, 1977). The role of SP in the transmission of acute nociceptive information is unclear. A decrease in the response of dorsal horn neurons to "brief" noxious cutaneous stimuli was observed by Radhakrishnan & Henry (1991) and De Koninck



& Henry (1991) using non-peptide NK<sub>1</sub> receptor antagonist CP,96345. However, the intensity and duration of the stimuli in those studies do seem substantial, and this compound exerts calcium channel-blocking effects, which should be considered when interpreting findings. In contrast, Fleetwood-Walker *et al* (1987; 1990), using spantide, saw little change in the responses of dorsal horn neurons to noxious cutaneous stimulation, but did observe an attenuation in those to innocuous stimuli.

In several experimental paradigms NK<sub>1</sub> receptor antagonists are seen to reduce nociceptive responses only after a sustained input or some form of conditioning stimulus: CP96345 (Xu *et al*, 1992) and RP67580 (another non-peptide antagonist, Laird *et al*, 1993) facilitate a nociceptive reflex following a noxious electrical conditioning stimulus, but have no effect on the baseline reflex. Also, while no effect of CP96345 is observed on single C-fibre volleys (De Koninck & Henry, 1991; Chapman & Dickenson, 1993), it does reduce the activity elicited in dorsal horn neurons during the second phase of the formalin response (Chapman & Dickenson, 1993) and high intensity electrical stimulation (De Koninck & Henry, 1991). Furthermore, when applied onto the spinal cord, SP enhanced and NK<sub>1</sub> antagonist [D-Pro<sup>2</sup>, D-Pro<sup>7,9</sup>]-SP attenuated wind up (Kellstein *et al*, 1990). In addition to this, RP67580 and CP96345 do not decrease responses to acute and sustained C-fibre stimulation in normal animals however they do following a UV-induced burn to the cutaneous receptive field (Thompson *et al*, 1994). Furthermore, flexion of the knee joint induces SP release in the dorsal horn, (measured with antibody microprobes), only following the induction of inflammation by an intra-articular injection of kaolin/carrageenan (Schaible *et al*, 1990). Capsaicin-induced sensitisation of primate STT neurons can be prevented by NK<sub>1</sub> antagonists CP96345 and GR82334 (Dougherty *et al*, 1994), though Nagy *et al* (1993) did not observe this in when recording from rat dorsal horn neurons in an *in vitro* spinal cord preparation. Thompson *et al* (1993) demonstrated an APV-resistant component of wind-up which is sensitive to both of the non-peptide NK<sub>1</sub> receptor antagonists mentioned above, but the peptide antagonist GR82334 failed to reduce the comparable sustained neuronal response elicited by applying mustard oil to the cutaneous receptive field (Munro *et al*, 1993). This may be an indicator as to the level of nociceptive input required before NK<sub>1</sub> receptors participate directly in nociception, since the collective evidence suggests NK<sub>1</sub> receptors play a more crucial role following a chronic nociceptive input.



## (b) Behavioural Studies

Although intrathecally administered SP evokes biting and scratching behaviours highly indicative of a painful sensation (Hayes & Tyers, 1979; Hylden & Wilcox, 1981; Yashpal *et al*, 1982) and it, or an NK<sub>1</sub> receptor agonist, can facilitate the tail flick reflex (Cridland & Henry, 1986; Gamse & Saria, 1986; Couture *et al*, 1993; Picard *et al*, 1993), non-peptide NK<sub>1</sub> antagonists fail to alter latencies to this acute noxious thermal stimulation of the tail, or paw (Garces *et al*, 1992; Malmberg & Yaksh, 1992a; Yamamoto & Yaksh, 1992a; Couture *et al*, 1993; Picard *et al*, 1993 - all using CP96345; Seguin *et al*, 1995 - CP99994, RP67580, SR140333, WIN51708 & WIN62577). Similarly in the hot plate test, SP does not cause facilitation (Gamse & Saria, 1986) nor does CP96345 prove antinociceptive (Garces *et al*, 1992).

Several antagonists to the NK<sub>1</sub> receptor do however appear to be analgesic when the noxious input is more prolonged. In writhing induced by acetic acid or phenylbenzoquinone, NK<sub>1</sub> receptor antagonists CP96345, CP99994, RP67580, SR140333, WIN51708 and WIN62577 are antinociceptive (Garret *et al*, 1991; Seguin *et al*, 1995). Similarly, acetylcholine-evoked abdominal constriction is reduced by CP96345, CP99994, RP67580 and peptide antagonist GR82334 (Birch *et al*, 1993). Furthermore, the behaviour induced by a prolonged noxious input due to the subcutaneous administration of formalin is reduced, particularly in the later phase, by CP96345, CP99994, RP67580, SR140333, WIN51708 and WIN62577 and by peptide antagonists GR82334 and D-Pro-SP (Garret *et al*, 1991; Murray *et al*, 1991; Yamamoto & Yaksh, 1991; Birch *et al*, 1992; 1993; Yashpal *et al*, 1993; Seguin *et al*, 1995). Nagahisha *et al* (1992) found both CP96345 and its enantiomer CP96344 (with low NK<sub>1</sub> affinity) to be antinociceptive in carrageenan- and formalin-induced behavioural responses, and although this has not been observed in all cases (see Yamamoto & Yaksh, 1991; Yashpal *et al*, 1993) data using only CP96345 should be considered with care. Capsaicin-induced licking and biting is also reduced by intrathecal injection of NK<sub>1</sub> antagonist D-Phe<sup>7</sup>-SP (Sakurada *et al*, 1992), as is heat injury-evoked hyperalgesia in the contralateral paw, with D-Pro<sup>2</sup>-SP (Coderre & Melzack, 1991). However, NK<sub>1</sub> receptor antagonists CP96345 and GR82334 failed to increase paw withdrawal latencies to noxious thermal or mechanical stimulation following chronic constrictive nerve injury- (Yamamoto & Yaksh, 1992a) or carrageenan- (Young *et al*, 1995a) induced hyperalgesia. The reason for such variability is likely to lie in the models, but could reflect distinct C-fibre populations.

### 1.4.3. Evidence for a Role of NK<sub>2</sub> Receptors in Sensory Processing

#### (a) Electrophysiological Studies

Unlike NK<sub>1</sub> receptors, there appears to be a clear role for NK<sub>2</sub> receptors in acute nociceptive transmission, as well as sustained. Selective NK<sub>2</sub> receptor antagonists, both peptide (D-Tyr<sup>4</sup>-SP and D-Pro<sup>4</sup>-SP) and non-peptide (SR48968), reduce neuronal responses to brief noxious thermal, but not mechanical, stimulation in both the dorsal horn (Fleetwood-Walker *et al*, 1990) and thalamus (Santucci *et al*, 1993). The baseline flexor reflex <sup>in muscle afferents</sup> can be facilitated by NKA and blocked by non-peptide antagonist MEN10376 (Xu *et al*, 1991). Similarly, single electrically-evoked C-fibre potentials can be reduced with MEN10376 both before (Thompson *et al*, 1993; 1994) and after a UV-induced burn to the cutaneous receptive field (Thompson *et al*, 1994). Also, pre-UV, UV-enhanced (Thompson *et al*, 1994), capsaicin-evoked (Nagy *et al*, 1993) or mustard oil-induced (Munro *et al*, 1993) sustained C-fibre inputs can be reduced by NK<sub>2</sub> antagonists MEN10376 and L659,874. Detection of IR-NKA, using by antibody microprobes, can be seen following noxious, but not innocuous, mechanical or thermal stimulation in the normal cat (Duggan *et al*, 1990). Furthermore, normally innocuous joint flexion fails to result in any release of IR-NKA until inflammation is induced by the injection of kaolin/carrageenan into the knee (Hope *et al*, 1990).

#### (b) Behavioural Studies

Few studies have addressed the antinociceptive role of NK<sub>2</sub> receptors by means of behavioural testing. Intrathecally injected NKA, or an NK<sub>2</sub> agonist, will decrease latencies in tail flick and, in some cases hot plate, test (Cridland & Henry, 1986; Gamse & Saria, 1986; Fleetwood-Walker *et al*, 1990; Couture *et al*, 1993; Picard *et al*, 1993). Accordingly, in the same tests, antagonists to NK<sub>2</sub> receptors, D-Pro-SP and L659,874 have been shown to increase latencies to such noxious thermal (though not mechanical) stimulation, both in naive and carrageenan-treated animals (Fleetwood-Walker *et al*, 1990; Young *et al*, 1995a; F.E. Munro, PhD thesis, 1995). Also, the second phase of formalin-induced behaviours can be inhibited by NK<sub>2</sub> antagonist SR48968 (Seguin *et al*, 1995). In contrast, this drug was not antinociceptive in the tail flick or acetic acid-induced writhing tests. Birch *et al* (1993) failed to see an antinociceptive effect of another NK<sub>2</sub> antagonist GR115211 in thermal (hot plate/tail flick) or chemical (acetylcholine-induced abdominal constriction/formalin-evoked biting and scratching) tests. However, in this study

GR115211 also failed to antagonise the nociceptive effect induced by intrathecally administered NK<sub>2</sub> agonist GR64349. As with behavioural data using NK<sub>1</sub> receptor antagonists the discrepancies may lie in subtleties in the method of testing, or indeed the drugs chosen.

The tachykinins seem to mediate nociception with temporal and modality differences through both NK<sub>1</sub> and NK<sub>2</sub> receptors. It would appear SP, via NK<sub>1</sub> receptors, is involved in mediating prolonged nociceptive inputs whereas NKA, through NK<sub>2</sub> receptors, mediates both brief and sustained nociceptive transmission, particularly of a thermal nature.

On some occasions the antinociceptive effects of NK<sub>1</sub> and NK<sub>2</sub> receptor antagonists can be altered, indeed enhanced, by the co-administration of EAA receptor compounds. It would appear that these two receptor groups interact in the dorsal horn to exert an integrated influence on dorsal horn neurons.

## **1.5. INTERACTIONS BETWEEN EXCITATORY AMINO ACID & TACHYKININ RECEPTORS**

Substance P and glutamate have been shown to co-exist in DRG cells (Battaglia & Rustioni, 1988) and, more importantly, C-, and some A $\delta$ -, afferent fibre terminals (DeBiasi & Rustioni, 1988) - suggesting that they are likely to be co-released upon stimulation of the presynaptic cell. Furthermore, since the presence of NKA almost always accompanies SP in DRG cells (Dalsgaard *et al*, 1985; see section 1.4.), all three neurotransmitters may be presumed to exist in the same primary sensory neurons. There is a growing body of evidence which suggests that glutamate and tachykinin receptors interact to enhance the transmission of nociceptive information.

### **(a) Electrophysiological Studies**

Both *in vitro* and *in vivo* studies support an interaction between NK<sub>1</sub>/NK<sub>2</sub> and glutamate receptors, with the general consensus being that agonists to neurokinin receptors can both enhance and attenuate responses induced by EAA receptor agonists. The release of EAAs can be induced by SP superfusion in both the frog (Nagy *et al*, 1981) and rat spinal cord, *in vitro* (Kangrga & Randic, 1990) and *in*

*vivo* (Skilling & Larson, 1993). Substance P was originally shown to enhance glutamate-evoked dorsal horn neuron activity in the 1970s (Zieglängsberger & Puil, 1973; Zieglängsberger & Tulloch, 1979). Since then several *in vitro* studies have reiterated this, but found also that EAA-evoked activity can be diminished by the co-application of SP: Randic *et al* (1990) found in voltage clamped isolated dorsal horn neurons that NMDA-, but not other EAA-, induced currents could be increased, or less often (in 35% of cells) decreased, following pretreatment with SP. Later Rusin *et al* (1992; 1993a,b) confirmed that the same occurred with NKA, though it caused a decrease in a higher proportion of cells (75% of those tested). The results due to co-administration versus pretreatment with tachykinins differed in that pretreating a neuron resulted in an initial decrease of NMDA-elicited responses, followed by an elevation, whereas a co-administration led to one or the other. *In vivo*, NMDA-evoked increases in responses of primate spinothalamic tract neurons to noxious mechanical stimulation of the receptive field can be augmented or attenuated when SP is simultaneously ionophoresed (Dougherty & Willis, 1991b; 1993), and in around half of the dorsal horn neurons in the cat tested by Song & Zhao (1994) the NK<sub>1</sub> antagonist spantide enhanced the reductions of C-fibre responses brought about by APV. Urban *et al* (1994) found NMDA or QA-induced ventral root potentials could be increased by both NK<sub>1</sub> agonist SP-methyl ester and NKA, but they did not report mixed effects.

Substance P-induced facilitation of the flexor reflex can be reduced with NMDA receptor antagonist MK801 (Xu *et al*, 1992), and in this study low doses of NK<sub>1</sub> and NMDA antagonists (MK801 and CP96345), that alone did not decrease this nociceptive reflex, reduced it markedly when given together. Similarly, the C-fibre-mediated activity elicited by capsaicin in DRG cells could be partially decreased by NMDA and NK<sub>2</sub> receptor antagonists, APV and MEN10376, but almost completely abolished when given together (Nagy *et al*, 1993). These studies are comparable with the NMDA- and tachykinin-sensitive components of wind-up that Thompson *et al* (1993) describe (see section 1.4.2.(a).)

## **(b) Behavioural Studies**

Kainate, AMPA and NMDA all evoke caudally-directed biting and scratching, but when the same dose is co-administered with SP the resulting behavioural responses are increased twenty-, thirty- and forty-fold respectively (Mjellem-Joly *et al*, 1991). Furthermore, while either NMDA or SP given alone as an intrathecal injection has no effect on formalin-evoked licking/biting of the

hindlimb, together they increase the response in both the acute and tonic phase (Mjellem-Joly *et al*, 1992). Also, SP- or noxious thermal stimulation-induced facilitation of the tail flick reflex is attenuated by NMDA receptor antagonist APV (Yashpal *et al*, 1991); results corresponding with those observed electrophysiologically by Xu *et al* (1992, see above). However, Murray *et al* (1991) did not observe any additive antinociceptive effect in the formalin test when antagonists to NK<sub>1</sub> and NMDA receptors were administered together.

Clearly there are various possible interactions between these receptors at the cellular level, and concurrent activation is likely to result in enhanced nociception.

The prolonged changes occurring due to a sustained noxious input cannot be fully attributed to the momentary effects induced by synaptic excitatory amino acid and/or neurokinin receptors. Instead they are likely to be mediated by the subsequent intracellular events triggered by the activation of such receptors.

#### **1.6. POSSIBLE SIGNAL TRANSDUCTION MECHANISMS INVOLVED IN NOCICEPTION**

When a ligand binds to a cell surface G-protein coupled receptor, such as the metabotropic glutamate or neurokinin receptors, it triggers another chemical signal (a second messenger) following the activation of the G-protein to which it is linked. This in turn leads to the engagement of further systems and is eventually manifest as a physiological result. Therefore such second messenger pathways govern the outcome of cellular activity by translating diffuse external information into limited internal signals.

Several recent studies have aimed to elucidate the intracellular mechanism(s) by which nociceptive information is transmitted in dorsal horn neurons. There are undoubtedly multiple second messenger pathways involved in this process, many of which are beyond the scope of this present study. There are five signal transduction targets which were investigated in the current experiments:

- Calmodulin/Ca<sup>++</sup>-Dependent Kinase II (CamKII)
- calcium-phospholipid-dependent protein kinase C (PKC)
- phospholipase A<sub>2</sub> (PLA<sub>2</sub>)



- cyclic AMP-dependent protein kinase (PKA)
- non-receptor tyrosine kinases

**(a) Calmodulin/ $\text{Ca}^{++}$ -Dependent KinaseII (CamKII)**

This is a multifunctional kinase which is activated, as its name suggests, by calcium and calmodulin, downstream of inositol-1,4,5-triphosphate ( $\text{IP}_3$ ) metabolism (see Figure 1.3.(a)). CamKII is abundant in neural tissue, and in the rat hippocampus it accounts for 2% of total protein (Erondy & Kennedy, 1985). It has been proposed that this kinase could act as a calcium-triggered molecular switch (Miller & Kennedy, 1986; Lisman & Goldring, 1988), with prolonged phosphorylated activity beyond the initial calcium-evoked activating signal. Such a role in prolonged synaptic events has already been investigated: the induction of LTP in the hippocampus can be prevented by application of CamKII inhibitors into the postsynaptic cell, though once the phenomenon has been initiated the inhibitor is no longer effective (Malenka *et al*, 1989; Malinow *et al*, 1989). Also, mutant mice which are deficient of CamKII fail to express LTP in the hippocampus, even though their behaviour appears otherwise normal (Silva *et al*, 1992). CamKII has been co-localised with glutamate receptors in the post synaptic density of forebrain synapses (Wu *et al*, 1986) and has been shown to modulate glutamate-induced currents *in vitro* in isolated Sf9 cells (McGlade-McCulloch *et al*, 1993) and in the snail (Watanabe & Onozuka, 1994) and crayfish (Noronha & Mercier, 1995). McGlade-McCulloch *et al* (1993) also reported how this kinase phosphorylated ionotropic glutamate receptors in cultured Sf9 and hippocampal cells. Together these data would suggest a regulatory role for CamKII in glutamate transmission.

**(b) Calcium/Phospholipid-Dependent Protein Kinase C (PKC)**

Phosphoinositide metabolism by phospholipase C results in the production of diacylglycerol (DAG) as well as  $\text{IP}_3$  (see Figure 1.3.(a)). DAG subsequently stimulates the translocation of protein kinase C (PKC) from the cytosol to the membrane (Manzoni *et al*, 1990), a recognised feature of its activation (Lutz *et al*, 1993). A role for PKC in synaptic plasticity has been strongly indicated. Inhibitors of this kinase will block LTP in the hippocampus (Lovinger *et al*, 1987; Malinow *et al*, 1988; 1989; Malenka *et al*, 1989), with their intracellular application demonstrating the particular importance of postsynaptic kinase activity for LTP induction (Malenka *et al*, 1989; Malinow *et al*, 1989). The exact role of PKC in LTP is unclear as some studies investigating the time course have reported that PKC becomes less crucial with time after initial LTP induction (Lovinger *et al*, 1987).

while others claim it is necessary for LTP maintenance (Reymann *et al*, 1988). A more recent discussion concluded that PKC activation alone seems insufficient to induce LTP (Bliss & Collingridge, 1993).

A role for PKC in the modulation of NMDA receptor currents has been reported in both the hippocampus (Ben-Ari *et al*, 1992) and the spinal cord *in vitro* (Gerber *et al*, 1989). Also, glutamate and aspartate release in the spinal cord slice can be increased with PKC activators (Gerber *et al*, 1989). Background activity and that evoked by innocuous cutaneous stimulation were both augmented by the infusion of PKC-activating phorbol esters (Palecek *et al*, 1994). Ionophoresis of selective PKC inhibitors markedly reduces the sustained activity in dorsal horn neurons elicited by the cutaneous application of C-fibre activator mustard oil (Munro *et al*, 1994a). This study and others, using a chronic constrictive injury (CCI, Mao *et al*, 1992; 1993) and formalin-evoked hyperalgesia (Yashpal *et al*, 1995), have shown by means of [<sup>3</sup>H]-phorbol-12,13-dibutyrate ([<sup>3</sup>H]PDBu) binding assays how sustained noxious inputs result in the translocation of PKC to the membrane compartment, in the spinal cord. Such PKC activation correlates with behavioural hyperalgesia (Mao *et al*, 1992; 1993). Furthermore behavioural hyperalgesia brought about by CCI (Hayes *et al*, 1992) or subcutaneous formalin, with or without glutamate-induced enhancement of the nociceptive input, (Coderre, 1992; Coderre & Yashpal, 1994; Yashpal *et al*, 1995) is reduced if PKC activation is prevented.

### (c) **Phospholipase A<sub>2</sub> (PLA<sub>2</sub>)**

This enzyme is responsible for the generation of arachidonic acid (AA) and ultimately its eicosanoid metabolites (see Figure 1.3.(a)) - prostaglandins (PG) and thromboxanes, via cyclooxygenase (COX - of which there are two forms - 'constitutive' COX1 (see Xie *et al*, 1992) and 'inducible', by inflammation, (Hla & Neilson, 1992) COX2), and leukotrienes via lipoxygenase. These metabolites are known to mediate numerous events in the periphery during inflammatory conditions. However, recent evidence suggests they may also play a crucial role centrally. Intrathecal treatment with AA results in the enhancement of behaviours elicited by the subcutaneous administration of formalin (Yashpal & Coderre, 1993). In addition to this, intrathecally administered non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the action of COX (McCormack, 1994), and AA inhibitors will markedly reduce the second, but not first phase of this formalin-induced behaviour. (Malmberg & Yaksh, 1992b; Coderre & Yashpal, 1994). The corresponding formalin-induced activity in dorsal horn neurons is also attenuated by intrathecal



NSAIDs (Chapman & Dickenson, 1992). Furthermore, the behavioural hyperalgesia induced by intrathecally applied NMDA, AMPA and SP can be decreased in a similar manner by NSAIDs (Malmberg & Yaksh, 1992a), as can SP- or glutamate-enhanced formalin responses be reduced by dexamethasone, an inhibitor of AA production (Coderre & Yashpal, 1994). Interestingly, in this study Coderre & Yashpal did not see any change in baseline paw withdrawal latencies when the inhibitor of AA was administered. Specific inhibition of COX2, by an antagonist selective over COX1, results in the reduction of carrageenan-induced hyperalgesia (Boyce *et al*, 1994).

**(d) Cyclic AMP-Dependent Protein Kinase (PKA)**

Following the activation of some receptors, adenylate cyclase mediates the formation of cyclic AMP (cAMP) from adenosine triphosphate (ATP). As a result of cAMP formation a cAMP-dependent protein kinase (PKA) is activated (see Figure 1.3.(b)). PKA is ubiquitous throughout the nervous system and is responsible for the phosphorylation of many intracellular proteins (Cohen, 1992). An NMDA receptor-dependent rise in cAMP in LTP has been reported (Chetkovitch *et al*, 1991), thus implicating a possible role for PKA in prolonged synaptic events. In isolated dorsal horn neurons the catalytic unit of PKA can increase NMDA-evoked currents, as can a cAMP analogue (Cerne *et al*, 1993), suggesting that this pathway may also be involved in regulating NMDA receptor sensitivity in the spinal cord.

**(e) Non-Receptor Tyrosine kinases**

As well as many membrane receptor tyrosine kinases (eg nerve growth factor (NGF) receptor, Kaplan *et al*, 1991), a variety of membrane-bound and cytosolic non-receptor tyrosine kinases are present in many cells (see Figure 1.3.(a)). These kinases have multiple tyrosine residues and are typically autophosphorylating (Cohen, 1992). The regulation of non-receptor tyrosine kinase activity with several IP<sub>3</sub>-linked G-protein receptors has been reported (Zachary *et al*, 1991). Extra- and

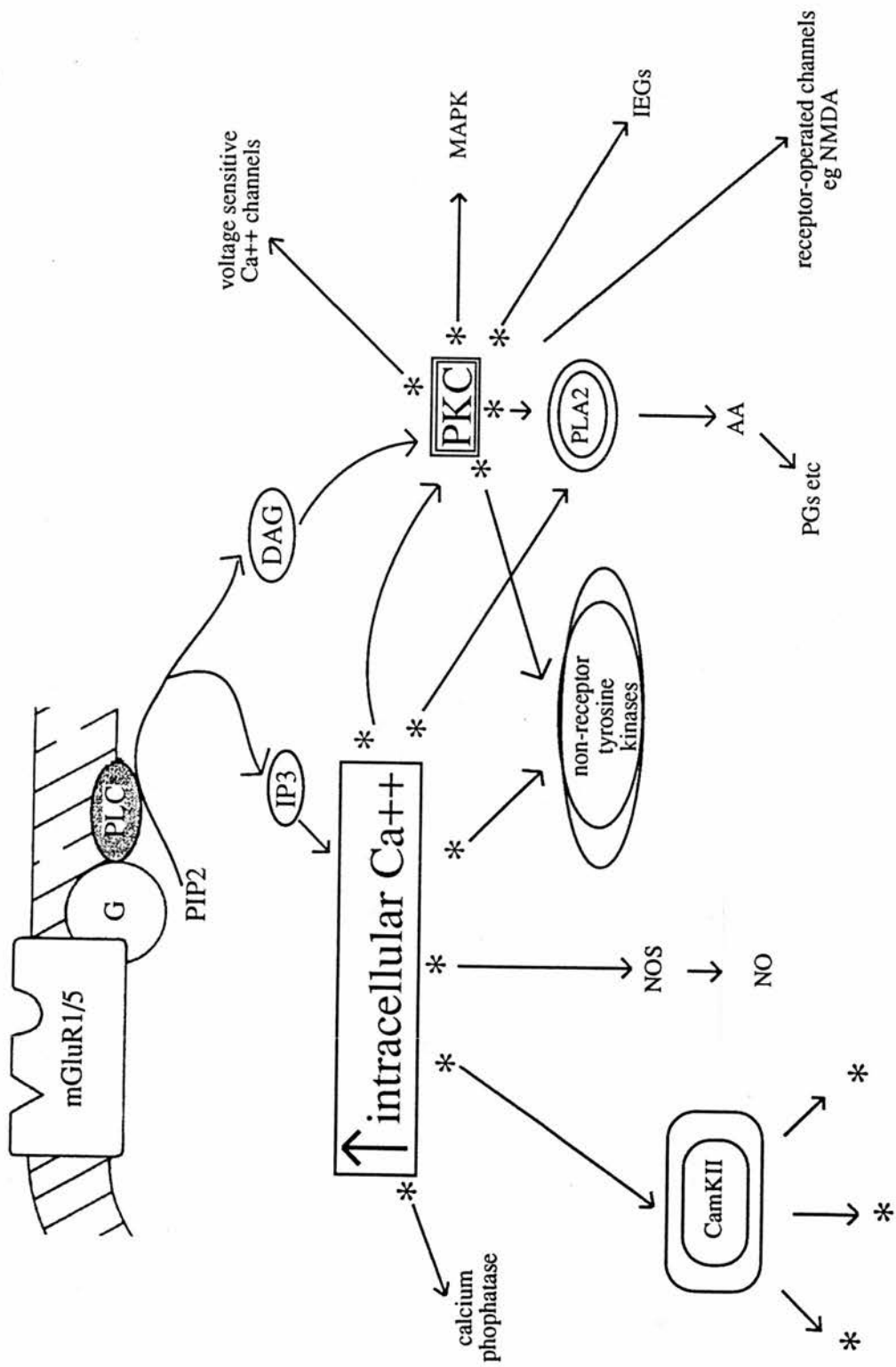
**Figure 1.3.(a)**

**Possible signal transduction following the activation of group I (1 and 5)  
metabotropic glutamate receptors**

Following the stimulation of metabotropic glutamate receptors 1 and 5 inositol phosphate and diacylglycerol activation can result in the triggering of many signal transduction pathways.

**AA** - arachidonic acid; **Ca<sup>++</sup>** - calcium; **CamKII** - calmodulin/Ca<sup>++</sup>-dependent kinase; **DAG** - diacylglycerol; **G** - GTP-binding protein **IEGs** - immediate-early genes; **IP<sub>3</sub>** - inositol -1,4,5-phosphate; **MAPK** - microtubule-associated protein kinase; **NO** - nitric oxide; **NOS** - nitric oxide synthase; **PGs** - prostaglandins; **PIP<sub>2</sub>** - phosphatidylinositol-4,5-biphosphate; **PKC** - protein kinase C; **PLC** - phospholipase C

\* - phosphorylation

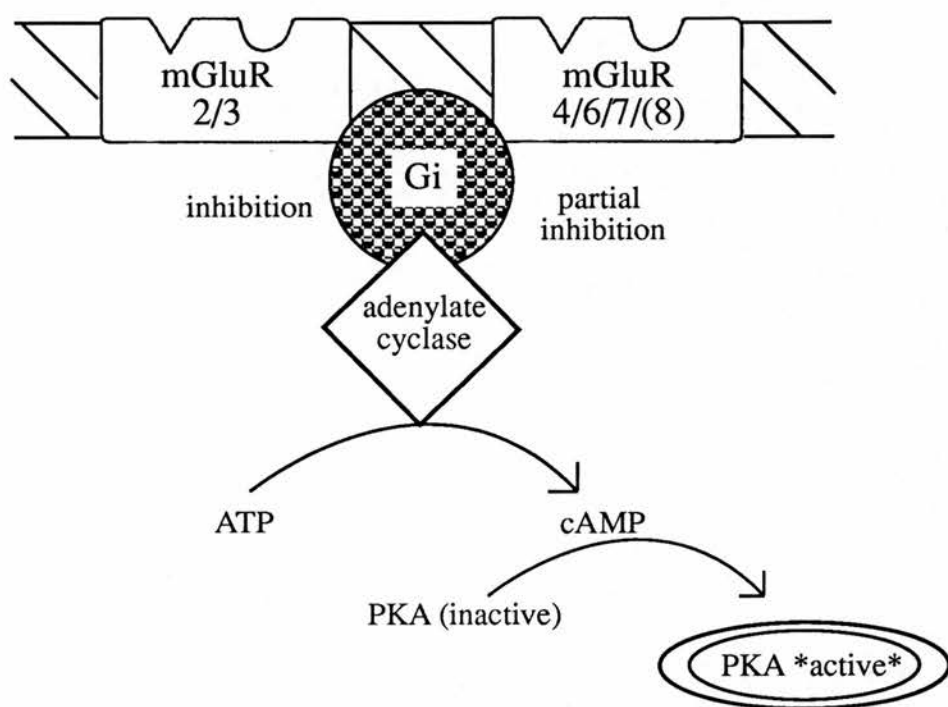


**Figure 1.3.(b)**

**Signal transduction following the activation of group II (2-4) and group III (6-8) metabotropic glutamate receptors**

Metabotropic glutamate receptors 2,3,4,6 and 7 (and 8) are linked to inhibitory G proteins (or partial, as shown) resulting in a decrease in the conversion of cAMP from ATP and subsequent lowering in the levels of active PKA.

**ATP** - adenosine triphosphate; **cAMP** - cyclic AMP; **Gi** - inhibitory GTP-binding protein; **mGluR** - metabotropic glutamate receptor; **PKA** - protein kinase A



postsynaptic intra- cellular application of tyrosine kinase inhibitors have been shown to block LTP, though they do not affect the phenomenon once it is established (O'Dell *et al*, 1991). They may therefore, by analogy,also play a role in sustained sensory inputs to the spinal cord.

Any or all of these signal transduction mechanisms may be involved in the transmission of nociception. As well as NK<sub>1</sub> and NK<sub>2</sub> receptors, group I mGluRs have been shown to operate through phospholipase C and therefore may act by mechanisms incorporating any of the downstream systems discussed. Beyond the initial effects of second messenger activation, a number of longer-term consequences are also initiated. The transcription of a number of genes, including immediate-early genes (IEGs) such as c-fos and c-jun ("third messengers"), is a further signal transducing mechanism within cells. Regulation of IEG function (both by increased expression and phosphorylation) is brought about by a variety of second messenger pathways, including some of those discussed, and is a critical factor in controlling expression of many cellular changes. There are many IEG products which may be involved in nociception, however this project concerns only the role of c-fos and its protein product Fos.

## **1.7. POSSIBLE MOLECULAR MECHANISMS INVOLVED IN NOCICEPTION**

Transcription factors, which are encoded by immediate-early genes (IEGs) such as proto-oncogenes c-fos and c-jun, are responsible for the initiation of gene expression in eukaryotic cells (Evan, 1991; Morgan & Curran, 1991). C-fos encodes a nuclear protein, Fos, suggesting that it could be involved in gene regulation. Heterodimeric transcription factors can be constructed from members of the c-fos and c-jun family, as can homodimers from the c-jun family alone, to interact with the activator-protein-1 (AP-1) DNA consensus sequence on target genes (Curran & Franza, 1988). The affinity for such consensus sequences depends on the fos-jun components of the transcription factor and both the relative levels of c-fos and c-jun (and their congeners) together with their phosphorylation can influence AP-1 activation. In cultured cells the activation of several second messenger pathways results in c-fos expression - these include calcium-calmodulin (Morgan & Curran, 1986), PKC (Greenberg *et al*, 1986) and cAMP (Greenberg *et al*, 1985) systems.

### 1.7.1. The Expression of c-fos as a Marker of Neuronal Activity In Response to Noxious Stimulation

Several studies using immunocytochemistry have demonstrated how Fos-like-immunoreactivity (c-fos LI; reflecting c-fos expression) can be elevated in response to a variety of noxious, but not innocuous, stimuli.

Fos-LI increases in the dorsal horn, primarily in the superficial laminae - the principal termination site for small nociceptive afferent fibres, following noxious thermal (Hunt *et al*, 1987; Bullitt, 1990; Tölle *et al*, 1990a, 1990b; 1994; Williams *et al*, 1990; Wisden *et al*, 1990; Dai *et al*, 1993; Abbadie *et al*, 1994), mechanical (Bullitt, 1989; 1991; Leah *et al*, 1992; Abbadie & Besson, 1994), chemical (mustard oil, Hunt *et al*, 1987; Pretel & Piekut, 1991) and electrical C-fibre strength (Herdegen *et al*, 1991; Molander *et al*, 1992) stimulation of the skin or the fibres innervating it. Histamine-evoked cutaneous itch also induces Fos-LI (Yao *et al*, 1992). Various non-cutaneous noxious stimuli have led to increased levels of c-fos expression in the dorsal horn: intramuscular mustard oil (Hunt *et al*, 1987), intra-articular urate crystals, which induce tissue damage, (Menétrey *et al*, 1989), intraperitoneal acetic acid, resulting in writhing, (Menétrey *et al*, 1989; Hammond *et al*, 1992; Lantéri-Minet *et al*, 1993a), and colorectal distension (Lanteri-Minet *et al*, 1993a; Traub *et al*, 1993) all increase Fos-LI. In contrast, innocuous brushing (Hunt *et al*, 1987; Bullitt, 1989), heating ( $\leq 44^{\circ}\text{C}$ ; Hunt *et al*, 1987; Wisden *et al*, 1990; Abbadie *et al*, 1994), or A $\beta$ -fibre strength electrical stimulation (Herdegen *et al*, 1991; Molander *et al*, 1992) of the cutaneous receptive field or nerve does not result in any increases in Fos-LI above those observed in controls. Molander *et al* (1994) found that although A $\beta$ -fibre strength electrical stimulation of the sciatic nerve did not result in any increase in Fos-LI in the dorsal horn of normal animals, it did 3-21 days after nerve transection - this may correspond with the abnormally painful sensations of A $\beta$ -fibre stimulation observed in allodynia (see section 1.3.1.(biii)).

Immunocytochemical and northern blot analysis has shown how sustained chemical stimulation also increases c-fos expression in the dorsal horn, again primarily the superficial laminae: intraplantar injection of carrageenan (Draisci & Iadorola, 1989; Ruda *et al*, 1990), CFA (Menétrey *et al*, 1989; Abbadie & Besson, 1992; Lantéri-Minet *et al*, 1993b) or formalin (Presley *et al*, 1990; Williams *et al*, 1990; Kehl *et al*, 1991; Leah *et al*, 1992; Sugimoto *et al*, 1994) leads to a rise in Fos-LI within as little as 15-30 minutes, usually peaking around 3-4 hours later. Interestingly, in an immunohistochemical study Zhang *et al* (1994) found that formalin-evoked c-fos expression could be abolished by capsaicin pretreatment (used to inactivate nociceptive fibres), suggesting that such expression is mediated, at least



partially, by C-fibres. Time-course experiments have shown how Fos-LI correlates with the behaviour seen in animals with CFA-induced acute inflammation and chronic arthritis (Abbadie & Besson, 1992; Lanteri-Minet *et al*, 1993b).

### 1.7.2. C-fos and Opioids

Pretreatment with morphine, or an opioid agonist, decreases the Fos-LI which is evoked in the dorsal horn by noxious thermal stimulation (Tölle *et al*, 1990a, 1990b; 1994; Abbadie *et al*, 1994), by intraplantar formalin administration (Presley *et al*, 1990), intraperitoneal acetic acid (Hammond *et al*, 1992) and by histamine-induced itch (Yao *et al*, 1992). One significant functional role of increased spinal c-fos expression elicited by nociceptive inputs is likely to be increasing the expression of genes with opioids which may be involved in endogenous analgesia. The augmented synthesis of these opioids evoked by noxious stimuli is preceded by an increase in c-fos expression in the ipsilateral dorsal horn (Draisci & Iadorola, 1989; Naranjo *et al*, 1991). Also, proenkephalin and prodynorphin mRNAs are colocalised with c-fos in some spinal neurons following inflammation (Noguchi *et al*, 1991; 1992). Furthermore, prodynorphin mRNA contains the AP-1 sequence (Naranjo *et al*, 1991), thus making it possible for Fos to bind and initiate transcription. These two groups of experiments with opioids underline the importance of c-fos expression in the control of other genes, and also exemplify how the measurement of c-fos expression can be used as a tool to assess the role of particular receptors in the dorsal horn in nociception/analgesia.

### 1.7.3. C-fos and Glutamate Receptors

Glutamate-, quisqualate-, kainic acid- and NMDA-induced c-fos expression has been reported in various central neurons including the hippocampus, both *in vitro* and *in vivo*, (Szekely *et al*, 1987;1989; Kaczmarek *et al*, 1988; Cole *et al*, 1989; Sonnenberg *et al*, 1989; Hisanga *et al*, 1992; Lerea *et al*, 1992). Morgan & Curran (1986) showed that an increase in intracellular calcium ( $[Ca^{++}]_i$ ) was required for c-fos activation. However voltage-gated  $Ca^{++}$  channel blockers do not affect NMDA-evoked c-fos expression (Szekely *et al*, 1989; Lerea *et al*, 1992), suggesting that this expression is mediated, at least in part, by NMDA receptor-linked  $Ca^{++}$  channels. However, the effect of NMDA receptor antagonists on c-fos expression is debatable. Some studies have found that pretreatment with MK-801 or APV can reduce noxious chemical-evoked c-fos expression in dorsal horn neurons

(Kehl *et al*, 1991; Birder & de Groat, 1992). Conversely others have failed to see any such reduction following noxious thermal stimulation with either these compounds or ketamine (Tölle *et al*, 1990a, 1990b; Wisden *et al*, 1990). These contrasting results may well be due to the nature of the noxious stimulus utilised. The possibility of metabotropic type glutamate receptors mediating nociception-induced c-fos expression has not been investigated.

## **1.8. AIMS OF CURRENT STUDY**

The present study aimed to elucidate the possible role of metabotropic glutamate receptors (mGluRs) in sensory processing, and the intracellular mechanisms by which they may elicit their effects. Also, an interaction between these receptors and NK<sub>1</sub> and NK<sub>2</sub> tachykinin receptors was investigated. This was done by means of electrophysiological, behavioural and molecular approaches and consists of three projects:

### **(a) Electrophysiological Experiments**

(a) In order to assess the role of mGluRs in individual cells, recordings from single dorsal horn neurons were made whilst they were activated by innocuous (quantified, motorised brush) or noxious (mustard oil) cutaneous stimuli, or by ionophoretic application of agonists specific to EAA receptors. Antagonists to mGluRs were ionophoresed during each of the evoked activities to determine (i) the role of these receptors in sensory processing and (ii) to verify antagonist specificity at metabotropic, not NMDA, glutamate receptors.

(b) Subsequent to the characterisation of mGluR involvement in sensory processing, the effect of specific inhibitors of several intracellular signalling pathways - (i) calmodulin/Ca<sup>++</sup>-dependent kinase II (CamKII), (ii) calcium-phospholipid-dependent protein kinase C (PKC), (iii) phospholipase A<sub>2</sub> (PLA<sub>2</sub>), (iv) cyclic AMP-dependent protein kinase (PKA) and (v) non-receptor tyrosine kinases - were investigated on brush-, mustard oil- and mGluR agonist-evoked activity. This was in order to determine the possible pathways involved not only in sensory processing, but also the activation of mGlu receptors.

**(b) Behavioural Testing**

In order to investigate the role of mGlu and neurokinin receptors in the whole animal, antagonists to each receptor were tested either alone or as part of a co-injection. Behavioural responses to noxious mechanical and noxious thermal stimulation of either (i) a normal paw, or (ii) in separate animals a paw which had undergone carrageenan-induced inflammation, were measured. The effect of intrathecal injections of (i) mGluR antagonists, L-AP3 or (S)-CHPG; (ii) NK<sub>1</sub> antagonist, GR82334; (iii) NK<sub>2</sub> antagonist, L659874; or (iv) a combination of each mGluR antagonist with either GR82334 or L659874 on paw withdrawal responses were investigated.

**(c) Molecular Biology**

A limited study employing a slot blot analysis of c-fos mRNA expression in the spinal cord was carried out to assess the role of mGluRs on a broad population of neurons (See Appendix I). Acute inflammation was induced in both paws by an intraplantar injection of complete Freund's adjuvant (CFA), a stimulus known to evoke c-fos expression in dorsal horn neurons. Simultaneously mGluR antagonists L-AP3 or (S)-CHPG were administered through an intrathecal cannula previously placed at the appropriate spinal segments. The whole spinal cord was examined for alterations in c-fos expression, in response to CFA with, and without, concurrent administration of mGluR antagonist.

## CHAPTER 2: MATERIALS

### 2.1. ANAESTHETICS

Urethane and  $\alpha$ -chloralose were obtained from Sigma Chemical Company, Poole, UK. Halothane was supplied by ICI Pharmaceuticals, Macclesfield, UK.

### 2.2. DRUGS

#### 2.2.1. Excitatory Amino Acid Receptor Agonists and Antagonists

(1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((**1S,3R**)-**ACPD**), 1-aminocyclobutane-*cis*-1,3-dicarboxylic acid (**ACBD**), L- and D-1-amino-3-phosphopropionic acid (**L- and D-AP3**), (R,S)-, (R)- and (S)-4-carboxy-3-hydroxyphenylglycine ((**R,S**)-, (**R**)- and (**S**)-**CHPG**) and (R,S)- $\alpha$ -methyl-4-carboxyphenylglycine ((**R,S**)- $\alpha$ -**MeCPG**) were all obtained from Tocris Cookson, Bristol, UK.

#### 2.2.2. Tachykinin Receptor Antagonists

[D-Pro<sup>9</sup>[spiro- $\gamma$ -lactam]Leu<sup>10</sup>,Trp<sup>11</sup>]physalaemin(1-11)(**GR82334**) was a gift from Glaxo Group Research, Ware, UK.

Acetyl-Leu-Met-Gln-Trp-Phe-Gly-NH<sub>2</sub> (**L659,874**) was supplied by Cambridge Research Biochemicals Ltd., Cambridge, UK.

#### 2.2.3. Second Messenger Inhibitors

3,4,3',5'-tetrahydroxy-trans-stilbene (**piceatannol**) and 5-{[2,5-Dihydroxyphenyl)methyl][(2-hydroxyphenyl)methyl]amino}-2-hydroxybenzoic acid (**lavendustin A**) were supplied from Boehringer Mannheim, Lewes, UK.

[2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (**ONO-RS-082**) was a gift from Dr Tsuboshima, ONO Pharmaceuticals, Osaka Japan.

**Benzenesulphonamide 4** was a gift from Mike Clark, Schering-Plough Research, Kenilworth, N.J., USA.

The remaining compounds were from Calbiochem Novabiochem Ltd., Nottingham, UK: 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl) maleimide (**GF109203X**), (1,2-dimethoxy-N-methyl-(1,3)benzodioxolo(5,6-C)phenanthridinium (**chelerythrine**), (1-[bis-(chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-[2,4-dichlorophenyl)methoxy]-ethyl-1H-imidazolium (**calmidazolium**), 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (**KN62**), N-[2-(p-bromom-cinnamylamino)ethyl]5-isoquinolinesulfonamide (**H89**), (8R,9S,11S)-(â)-9-hydroxy-9-(n)hexanoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cycloocta[c,d,e]trinden-1-one (**KT5720**).

### 2.3. OLIGOMER PROBES AND RADIOLABEL

The oligodeoxyribonucleotides used were synthesised and purified by high performance liquid chromatography by Oswel Chemicals, Department of Chemistry, University of Edinburgh, UK. Deoxyadenosine [ $\alpha$ - $^{35}\text{S}$ ]-triphosphate, used to label the oligomer probes, was supplied by New England Nuclear Research Products, Du Pont de Nemours, Dreiech, Germany. The specific activity of the radiolabel was  $1\text{--}1.3 \times 10^3$  Ci/mmol. It was stored at  $-70^\circ\text{C}$  until required. Terminal deoxynucleotidyl transferase and potassium cacodylate tailing buffer, used in labelling the probes, came from GIBCO BRL Ltd., Paisley, UK. Nu-Clean D25 disposable spun columns were from International Biotechnologies Inc., New Haven, Connecticut, USA.

### 2.4. MISCELLANEOUS

Allyl isothiocyanate (**mustard oil**) was obtained from Aldrich Chemical Company, Gillingham, England. Liquid paraffin was supplied by Mackay & Lynn, Edinburgh, UK. Lambda **carrageenan** and **complete Freund's adjuvant** were both from Sigma Chemical Company, Poole, UK.

All reagents were of highest analytical grade and, unless otherwise stated in the text, were obtained from Sigma Chemical Company, Poole, UK.

## CHAPTER 3

### THE EFFECTS OF METABOTROPIC GLUTAMATE RECEPTOR ANTAGONISTS ON RESPONSES OF DORSAL HORN NEURONS TO SENSORY STIMULI AND TO GLUTAMATE RECEPTOR AGONISTS

#### 3.1. AIMS

The effects of several antagonists of metabotropic glutamate receptors (mGluRs) on dorsal horn neurons were investigated comparing activity evoked by (i) innocuous brushing of the cutaneous receptive field and (ii) noxious chemical stimulation by topical application of mustard oil to the skin. The pharmacological specificity of one compound, L-AP3 and its stereoisomer, D-AP3 was also assessed using agonists to mGlu and NMDA receptors.

#### 3.2. METHODS

##### 3.2.1. Animals

Wistar rats were purchased from Charles River UK Ltd, Margate, Kent, UK. They were housed in groups of 3 or 4 in standard rat cages on a 12 hour light/12 hour dark cycle, with a room temperature of 19-23°C. Food and water were available *ad libitum*.

##### 3.2.2. Surgical Procedures

Experiments were carried out on adult males (240 - 420g). Under an initial halothane anaesthesia the jugular vein and trachea were cannulated. Animals were then given intravenous  $\alpha$ -chloralose (60mg kg<sup>-1</sup>) and urethane (1.2g kg<sup>-1</sup>). Supplementary doses of 0.2ml (25mg ml<sup>-1</sup>)  $\alpha$ -chloralose were given throughout the experiment, requirement determined by blink reflex. Core body temperature was maintained at 37-38°C by means of a thermostatically-controlled heat blanket linked to a rectal probe. The animal was allowed to breathe freely with a light flow of oxygen (0.1l / min) passed over the tracheal cannula to enrich the inspired air.

The animal was placed in a stereotaxic frame, with stability provided by jaw and ear bars. A pool was made by tying back the skin flaps. The thoraco-lumbar spinal column was supported using 3 pairs of swan-necked clamps, the middle clamp being assigned to the position of the floating rib (in the region of T12 spinal process and spinal segment L2). A laminectomy, L1 - L4, was performed under x12.5 magnification. To provide further stability, agar (2% in 0.9% saline, 39°C) was

injected under the most rostral vertebra and then over the whole pool, including the spinal cord. Once the agar was set a small area was removed to uncover the region of interest for recording. Finally the dura was removed, ensuring pressure was relieved at both ends of the cord. Liquid paraffin, 37°C, was then poured over the exposed spinal cord to prevent dehydration.

### **3.2.3. Electrophysiological Recording and Ionophoresis of Drugs**

Extracellular recordings were made from single neurons at depths of 0-1000µm (as stated on the microdrive) via the central barrel of a 7-barrelled glass microelectrode filled with 4M NaCl (pH 4.0-4.5). The tip diameter ranged from 4-5µm with dc resistances of 5-8MΩ. The bandwidth of the recording amplifier was 1Hz - 7kHz. The microelectrode was secured by means of a steel arc which spanned the animal transversely, and moved through the spinal cord in 2 or 6µm steps using a microdrive system. Extracellular recordings from the central barrel was displayed on an oscilloscope (Tektronix). Output activity from the oscilloscope was continuously monitored using a D.130 Digitimer Spike Processor. Neuronal firing rate, along with the analogue signal from the heat stimulator, was continuously plotted on-line by a customised analysis program on an IBM PS/2-70-121 computer (Scap 90, Dr M. Dutia, Department of Physiology, University of Edinburgh). This program allowed event markers to be added as data was collected. Occasionally recordings onto FM tape were also made.

Drugs were ionophoresed from the side barrels of the electrode. Retention currents of ±10nA were employed to minimize drug leakage between tests. One of the remaining barrels contained 1M NaCl (pH 4.0 - 4.5) for automatic current balancing, using a Neurophore BH2 Ionophoresis system (Medical Systems Corporation), and current controls. A second side barrel contained Pontamine Sky Blue (PSB; 2% in 0.5M sodium acetate) which was used to mark recording sites by ejecting for 12-14 minutes at 10µA. The resistance of side barrels was standardly measured and ranged from 20-30MΩ.

### **3.2.4. Drugs for Ionophoresis**

All solutions were 10mM in distilled water, pH adjusted to 8.0-8.5 with sodium hydroxide.

*Agonists* - Metabotropic glutamate receptor: (1S,3R)-ACPD (Irving *et al*, 1990) and NMDA receptor: ACBD (Lanthorn *et al*, 1990)

*Antagonists* - Metabotropic glutamate receptor: L-AP3 (Schoepp *et al*, 1990a), (R,S)-CHPG (Watkins *et al*, 1987), (S)-CHPG (Glaum *et al*, 1993) and



(R,S)- $\alpha$ -MeCPG (Eaton *et al*, 1993; Pook *et al*, 1993). D-AP3, a weak NMDA antagonist (Schoepp *et al*, 1990a).

### **3.2.5. Controls**

1M NaCl was ionophoresed upto 80nA for simultaneous current and vehicle controls. These controls were carried out on agonist-, brush- and mustard oil-evoked activity.

### **3.2.6. Quantification of Neuronal Responses to Cutaneous Sensory Stimuli**

Neurons were located by simultaneously lowering the electrode into the spinal cord, using a microdrive, and manually brushing the hindlimb. All neurons were multireceptive and had cutaneous receptive fields located on the ipsilateral hindlimb. Care was always taken to ensure clear discrimination of spikes from any other activity (see Figures 3.2 and 4.7). Once the most appropriate position for the electrode was found, some background firing was recorded, then neuronal characterization was carried out by manual innocuous brushing and noxious pinching and by quantified noxious heating. The heat stimulus was supplied by a thermistor-controlled radiant heat lamp which was placed on the skin and raised from 30°C to 46-48°C in 5 seconds, and was maintained for a total of 10 seconds. Test innocuous brush stimulation was provided by positioning a rotating motorised brush onto the centre of the receptive field for the duration of drug ionophoresis, as well as one minute prior to and one minute after the application of drugs.

### **3.2.7. Quantification of Neuronal Responses to Cutaneous Application of Mustard Oil**

Neurons were located as described above. Mustard oil is a chemical irritant which can be administered topically, intramuscularly or intra-articularly in order to selectively activate C-afferent fibres (Woolf & Wall, 1986; Heapy *et al*, 1987). Although it does activate some A $\delta$  fibres, the reponse they exhibit is transient compared to that of C-fibres (Heapy *et al*, 1987; Harris & Ryall, 1988). Similarly, C afferents innervating hairy, rather than glabrous, skin are preferentially activated by this chemical (Harris & Ryall, 1988), this may purely be due to skin penetration, but could also reflect a group of mustard oil-insensitive C-fibres. Mustard oil induces skin temperature increases of 2-3°C, an increase in plasma extravasation and oedema, all of which are thought to be a result of an increased release of vasoactive mediators from capsaicin sensitive fibres (Lippe *et al*, 1993). When applied to abraded skin in man it causes an intense burning sensation (Woolf, 1984) and it results in a prolonged flexion reflex in the rat (Woolf & Wall, 1986). Mustard oil

induces central sensitisation (Woolf & Wall, 1986), one of the manifestations of which is an increase in the cutaneous receptive field (Woolf & King, 1990). The cutaneous application of mustard oil in this study was used to evoke a sustained level of C-fibre activation, analogous to that seen in "wind-up" (see section 1.3.1.(biii)) Since the vigorous response of a neuron to noxious heating of the receptive field is a good indication of C-fibre innervation, a cell responding to noxious heat stimulation, which was not on the glabrous skin, was considered acceptable for mustard oil application. Occasionally, cells in which noxious pinching, but not heating, evoked a response were tested for a response to mustard oil; this rarely resulted in elevated firing.

Following the standard background recording, mustard oil (7.5% in liquid paraffin) was topically administered to the receptive field using a small paint brush. Sometimes one application was sufficient to evoke a maintained level of neuronal activity. However, if it was not, applications were repeated at 5 minute intervals until a sustained level of elevated neuronal firing was observed. No tests were executed until the response was stable for 1-2 minutes. Therefore drugs were tested at anything between 5 and 35 minutes after the first administration of mustard oil.

Initially compounds were ionophoresed for 1-2 minutes. This time was adjusted depending on their effect on the neuron, some drugs were ejected for up to 6 minutes in later tests.

### **3.2.8. Quantification of Neuronal Responses to Ionophoretic Application of Agonists**

Cells were found in one of two ways: either in the usual manner, whereby they would be located due to manual brushing of the hindlimb, or by searching with the agonist on an ejection current of 5-10nA. If they were found by brushing the hindlimb, the agonist would be ionophoresed following at least 1 minute of recorded basal firing. Once a stable response to the agonist was obtained, an antagonist was co-ejected. If a cell was located using the other method, ionophoresis of the agonist was terminated to ensure neuronal firing stopped as a result. Basal firing would be recorded in the usual way and tests resumed as above.

### **3.2.9. Histological identification of recording sites**

At the end of the majority of experiments the spinal cord was removed in order to determine the position of the recording electrode. The cord was rostro-caudally marked then fixed in 10% formal saline. It was then mounted on a freezing

microtome in 0.25% agar and 52 $\mu$ m sections were cut until a blue spot, formed in the experiment by ejecting PSB at 10 $\mu$ A for 12-14 minutes, could be observed. The sections containing this blue dye were then placed onto gelatin-subbed slides and stained with 1% neutral red. Observation under a light microscope allowed the laminar position of the electrode to be defined.

### **3.2.10. Analysis of Results**

Analysis was carried out off-line using the Scap 90 program. The inhibitory effects of antagonists on evoked activity (agonists, mustard oil and motorised brush) were calculated by comparing mean firing levels immediately prior to their administration (10-60seconds) with mean activities during their iontophoresis. The period of greatest inhibition (10-60seconds) was chosen for such comparisons, and expressed as a percentage of the control value taken before antagonist administration. All values are given as the mean  $\pm$  standard error of the mean (SEM). The statistical significance of drug-induced changes from control responses was evaluated using the Wilcoxon test.

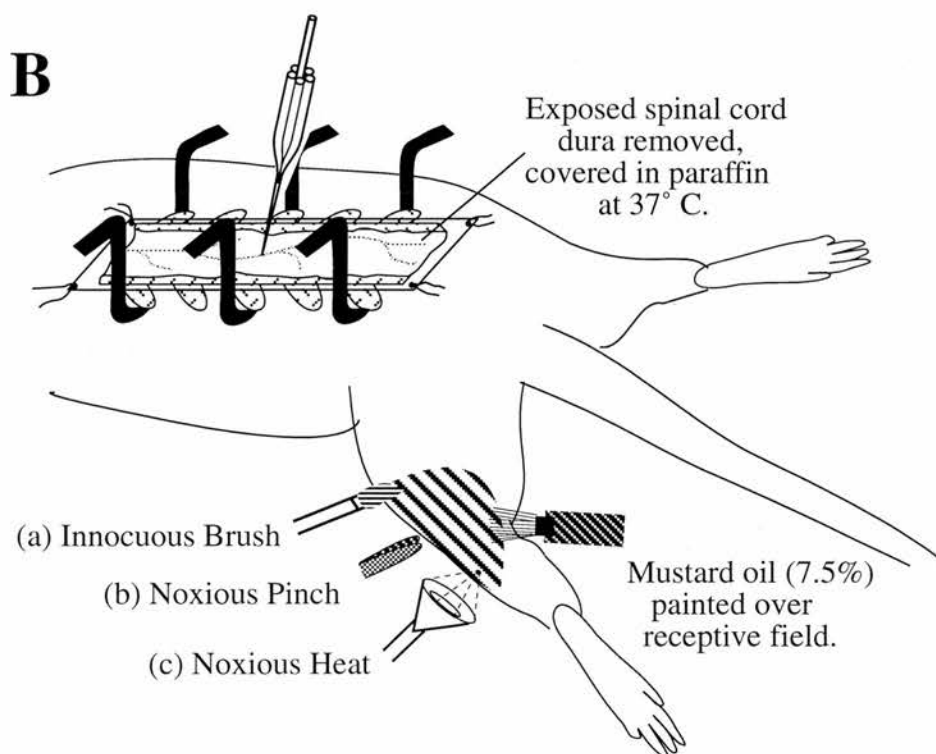
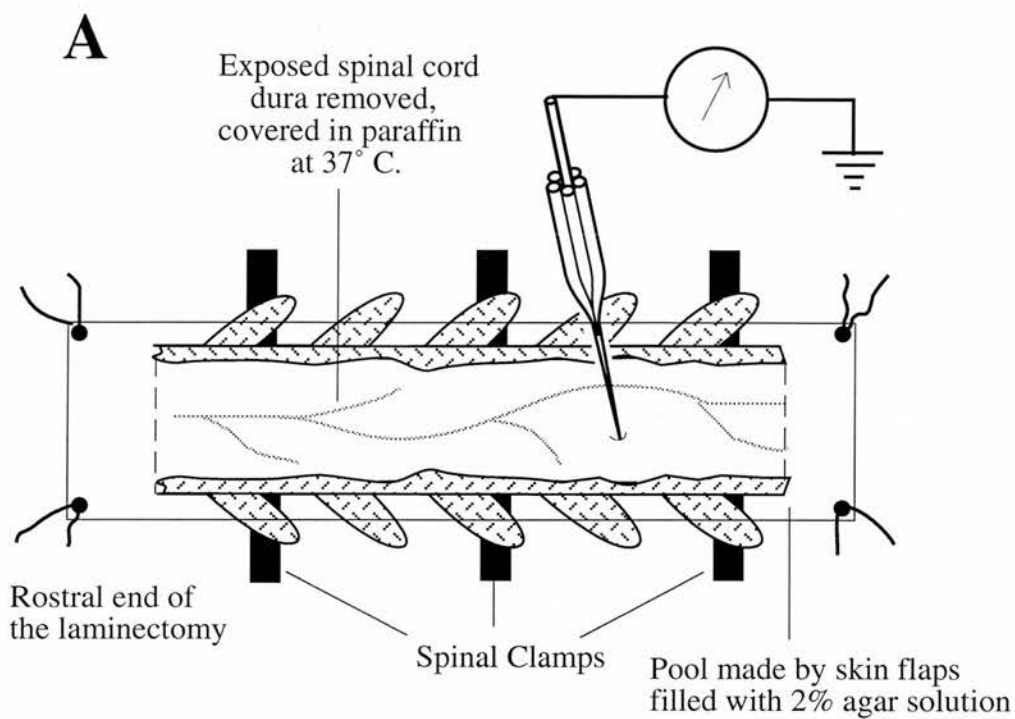
Changes brought about in neuronal firing which were within 15-20% of pre-drug controls were not considered to be significant, as they were within (or close to) the range of natural variability brought about by vehicle or current controls.

**Figure 3.1.**

**Schematic representation of the protocol utilised in electrophysiological recording experiments**

**A:** Schematic representation of the spinal cord in the region of L1 - L4, revealed by a laminectomy performed on vertebrae stabilised using 3 pairs of swan-necked clamps. Following this exposure, the spinal cord and surrounding area was covered with 2% agar, to provide stability during recording. Upon removal of an agar core immediately above the exposed cord, 37°C paraffin was applied and extracellular recordings were made via a 7-barrelled glass microelectrode.

**B:** During extracellular recordings activity was evoked in dorsal horn neurons by various peripheral stimuli. Activity was elicited with (a) a motorised rotating brush, (b) calibrated pincher and/or (c) a radiant heat lamp. Noxious chemical stimulation was attained by topically applying 7.5% mustard oil onto the receptive field. Finally, activity in dorsal horn neurons could be induced by ionophoresing the metabotropic glutamate receptor (1S,3R)-ACPD from a side barrel of the glass microelectrode. Various antagonists were applied in a similar manner.



### 3.3. RESULTS

#### 3.3.1.1. Characteristics of Neurons

In all, 90 neurons were investigated, from 47 rats, with microdrive electrode depth readings of 150-1000 $\mu$ m from the spinal cord surface. Neurons consistently had receptive fields on the cutaneous surface of the ipsilateral hindlimb. The 'receptive field' was defined as the area in which a response to innocuous brushing was observed, this always incorporated the receptive field to noxious pinch or heat, which tended to be smaller. Basal firing rates were low (0-1Hz).

Of the 22 neurons investigated for the effect of antagonists on innocuous brush-evoked activity, not all neurons were characterised, but in the total population, examples of multireceptive and low threshold non-nociceptive cells were observed. No nocispecific cells were examined; most likely due to the search strategy, in which cells were initially found by manual brushing of the hindlimb. All of the cells assessed here responded readily to innocuous stimuli, and a number of them also displayed nociceptive responses. One out of four cells tested responded to noxious heat (46- 48°C, 10 seconds), 10 of 11 tested responded to noxious pinching (manual), 3 units were activated by brush only and 7 were not characterised in terms of their sensory inputs.

A total of 28 neurons were used in the mustard oil experiments, all of which were multireceptive and responded vigorously to noxious heating of the cutaneous receptive field. Experience indicated a greater likelihood of the cell responding with a sustained increase in activity to mustard oil application, if it had responded to the noxious heat stimulus. In later experiments, when changes in the receptive field were monitored, it was noted that the application of mustard oil most frequently caused an increase in its size. Such alterations have been seen before with this chemical and are suggestive of central neuronal sensitisation (Woolf & Wall, 1986)

For the agonist study a total of 40 neurons were assessed. Of the units responding to (1S,3R)-ACPD or ACBD, 9 out of 12 and 7 out of 12 tested respectively also fired when their receptive field was stimulated with noxious heat. In 20 cells tested with both agonists, 6 gave clear responses to both, 3 responded selectively to (1S,3R)-ACPD, 9 to ACBD only and 2 were unaffected. In each category there were examples of cells which did or did not respond to heat. The

approximate dorsoventral positions of these cells, with their responses to agonists and heat can be seen in Fig 3.7. No correlation was found between depth and the presence/absence of a thermal nociceptive input or responsiveness to agonists.

### **3.3.1.2. Current Controls**

Although the equipment employed had an automatic current balancing system, therefore minimising the likelihood of current effects on cell membranes, some additional controls were performed. The ejection of 1M Na<sup>+</sup> did not depress the firing of the cells studied. In the absence of current balancing extracellular cation ejection would be expected to hyperpolarise adjacent cell membranes.

### **3.3.2. EFFECTS OF MGLU RECEPTOR ANTAGONISTS ON NEURONAL RESPONSES TO INNOCUOUS BRUSH STIMULATION**

Firing was evoked by placing a motorised rotating brush applied to the central area of the cutaneous receptive field. One minute of this evoked activity (8-45Hz) was recorded before applying any compounds. Three mGluR antagonists were assessed (see Fig 3.3): L-AP3, (R,S)-CHPG and (S)-CHPG, the isomer selective for mGluRs, as opposed to the NMDA-type glutamate receptor (Birse *et al*, 1993; Glaum *et al*, 1993). No significant change ( $p > 0.05$ , Wilcoxon test) was brought about in neuronal firing for either L-AP3 (9 out of 9 cases), (R,S)-CHPG (5 out of 5) or for (S) CHPG (12 out of 12). Occasionally during the iontophoresis of these compounds a slight increase in activity was observed, but this was never greater than 15% of the brush-evoked response, was not consistent and was always transient. All three drugs were iontophored from -15 to -55nA.

### **3.3.3. EFFECTS OF MGLU RECEPTOR ANTAGONISTS ON NEURONAL RESPONSES TO NOXIOUS CHEMICAL STIMULATION BY MUSTARD OIL**

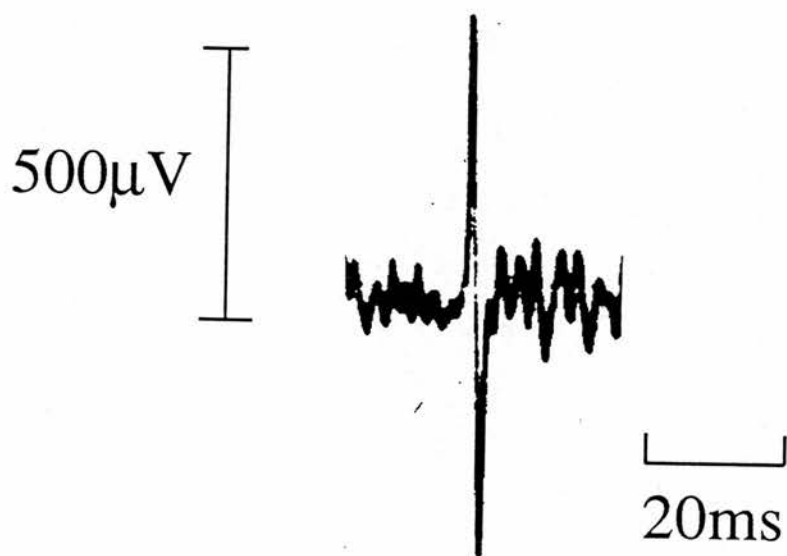
Mustard oil-evoked activity (5-13Hz) was markedly inhibited by L-AP3 (-15 to -50nA), by  $68 \pm 7\%$  (mean  $\pm$  SEM;  $p < 0.05$ , Wilcoxon test on raw data), in 11 out of 16 cells. In contrast, its stereoisomer D-AP3, in the same current range, failed to affect such firing. (R,S)-CHPG (-15 to -55nA) also caused clear reductions in firing in 7 out of 8 units, by  $56 \pm 9\%$  ( $p < 0.05$ ) Typical examples



**Figure 3.2.**

**Example of an action potential recorded from a single dorsal horn neuron**

This oscilloscope record shows a typical example of a clearly discriminated action potential.

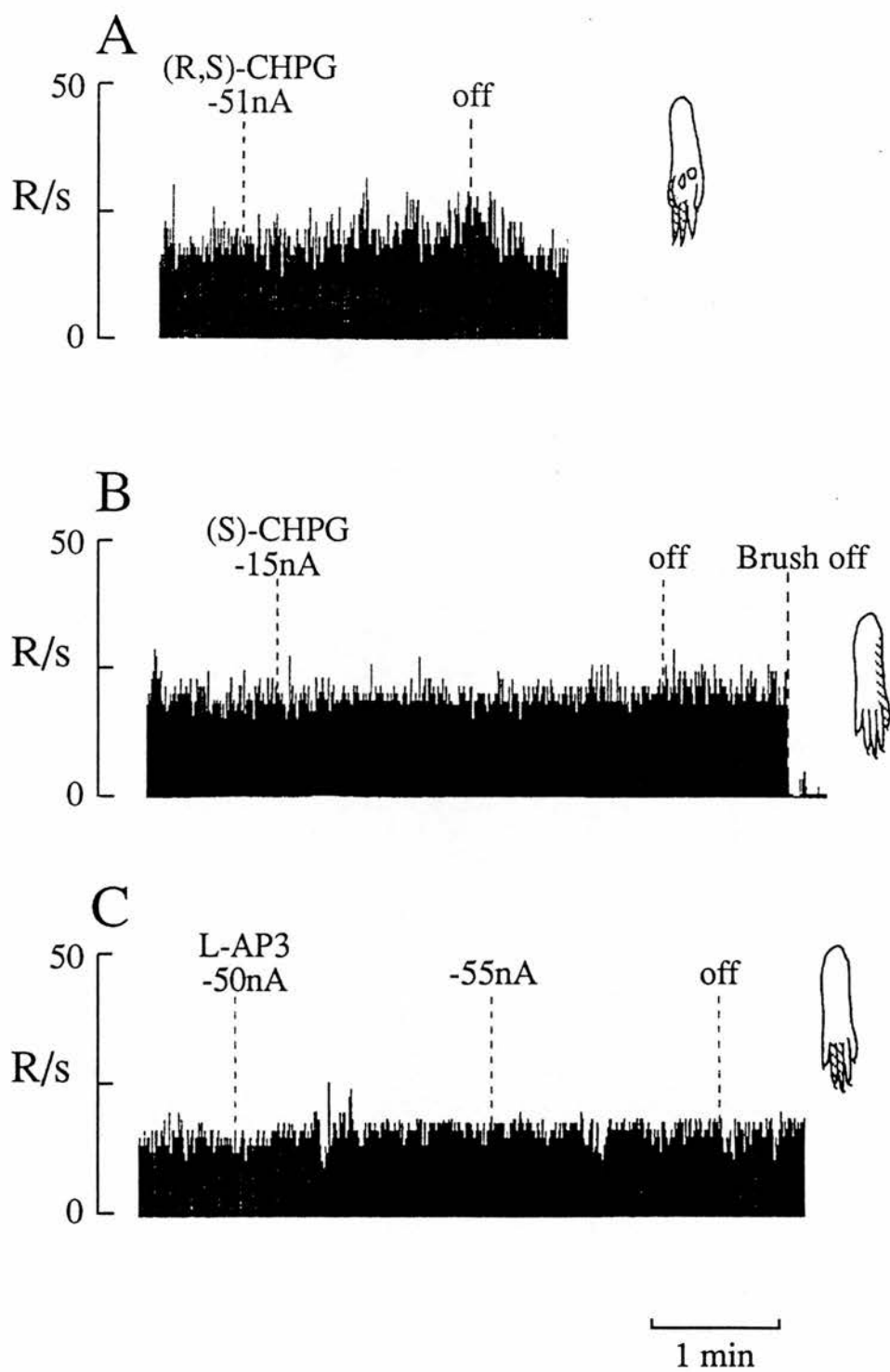


### **Figure 3.3**

#### **The effect of ionophoretically applied metabotropic glutamate receptor antagonists on brush-evoked activity in dorsal horn neurons**

The records show ongoing firing of individual neurons, displayed as action potentials per second (R/s), integrated over 700 millisecond bins, plotted against time. The hatched areas on the inserts show the receptive field of the cells.

Each neuron was activated by motorised brushing of the cutaneous receptive field. All three metabotropic glutamate receptor antagonists failed to significantly inhibit such brush-evoked firing, at the currents shown. In the cases of (R,S)-CHPG and L-AP3, these currents used for antagonists were greater than or equal to those known to reduce mustard oil-evoked responses.

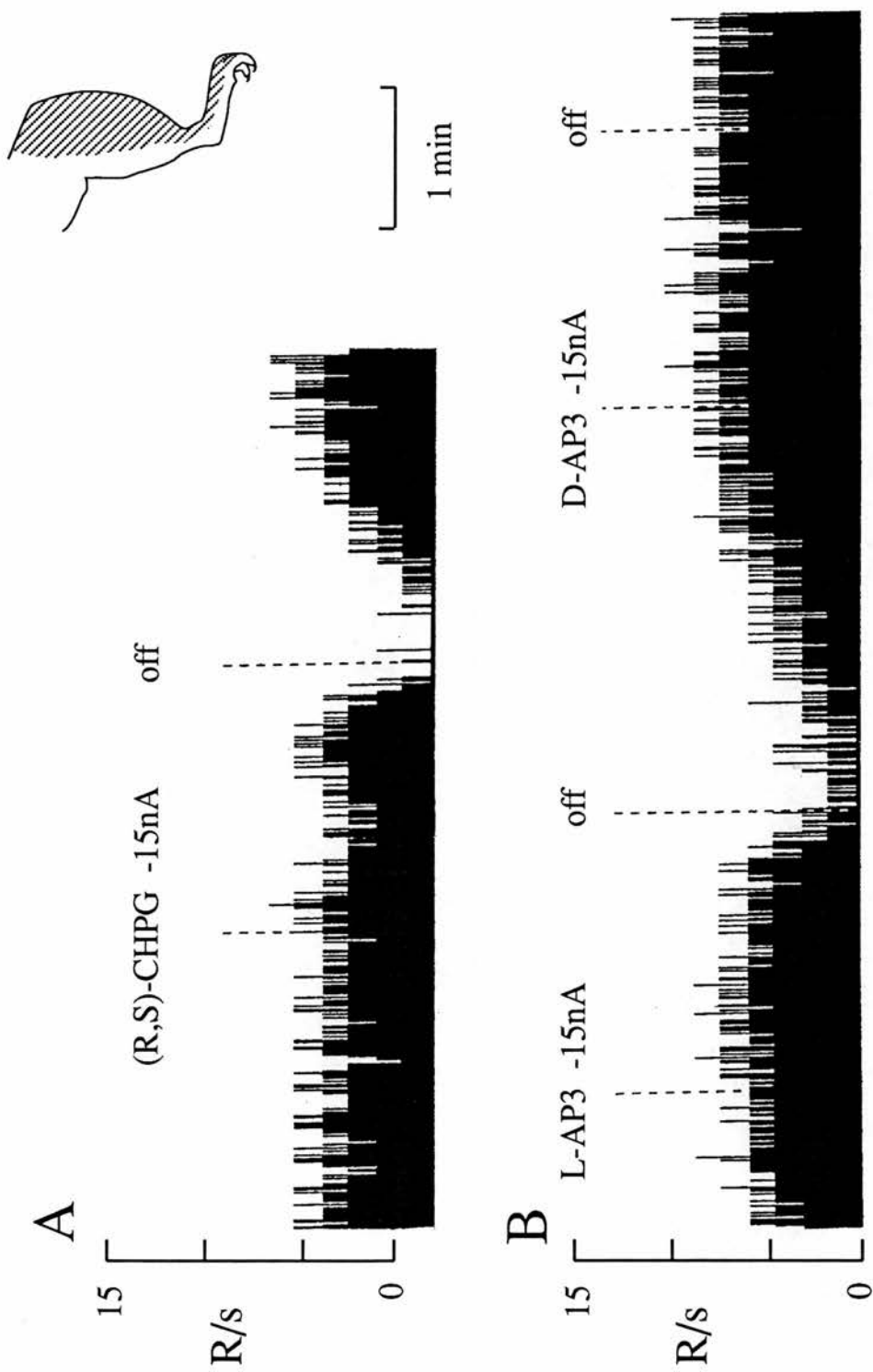


### **Figure 3.4**

#### **The effect of ionophoretically applied metabotropic glutamate receptor antagonists on mustard oil-evoked activity in dorsal horn neurons**

The records show ongoing firing of an individual neuron, displayed as action potentials per second (R/s), integrated over 700 millisecond bins, plotted against time. The receptive field of the cell is represented by the hatched areas in the insert.

The traces show activity induced by the application of mustard oil to the cutaneous receptive field. Metabotropic glutamate receptor antagonists (R,S)-CHPG and L-AP3 both significantly inhibited this firing ( $p < 0.05$ , Wilcoxon test). However, D-AP3, the stereoisomer of L-AP3, did not alter mustard oil-evoked firing when ionophoresed at equivalent currents.



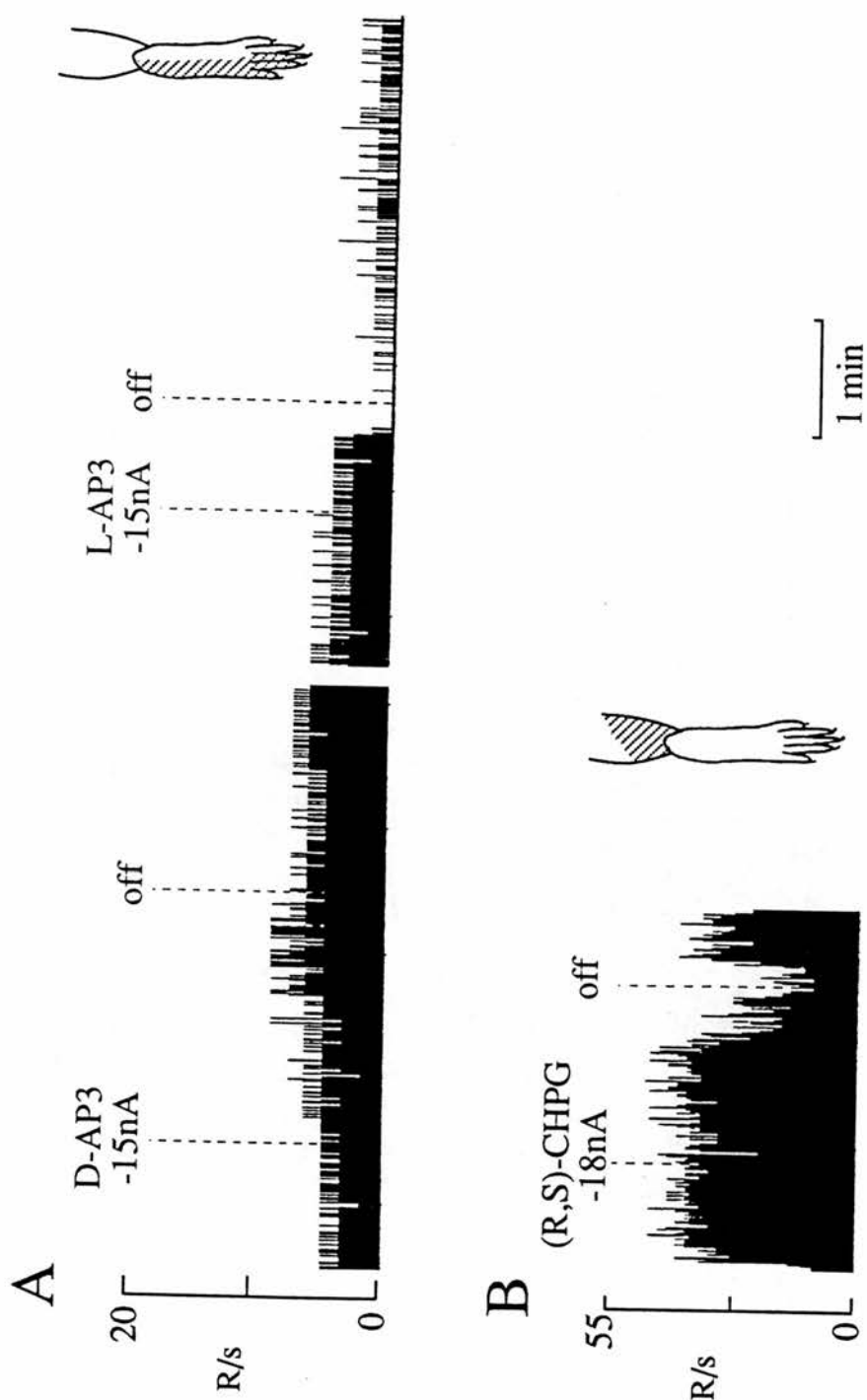
### Figure 3.5

#### **The effect of ionophoretically applied metabotropic glutamate receptor antagonists on (1S,3R)-ACPD-evoked activity in dorsal horn neurons**

The records show ongoing firing of individual neurons, displayed as action potentials per second (R/s), integrated over 700 millisecond bins, plotted against time. The hatched areas on the inserts show the receptive field of the cells.

Record A shows how L-AP3, but not D-AP3, reduced firing evoked by metabotropic glutamate receptor (mGluR) agonist (1S,3R)-ACPD (applied at -1-2nA prior to and during the test). In B a second mGluR antagonist, (R,S)-CHPG, can also be seen reducing agonist-evoked activity. The currents at which both antagonists were applied are equivalent to those which reduced firing induced by the cutaneous application of mustard oil.





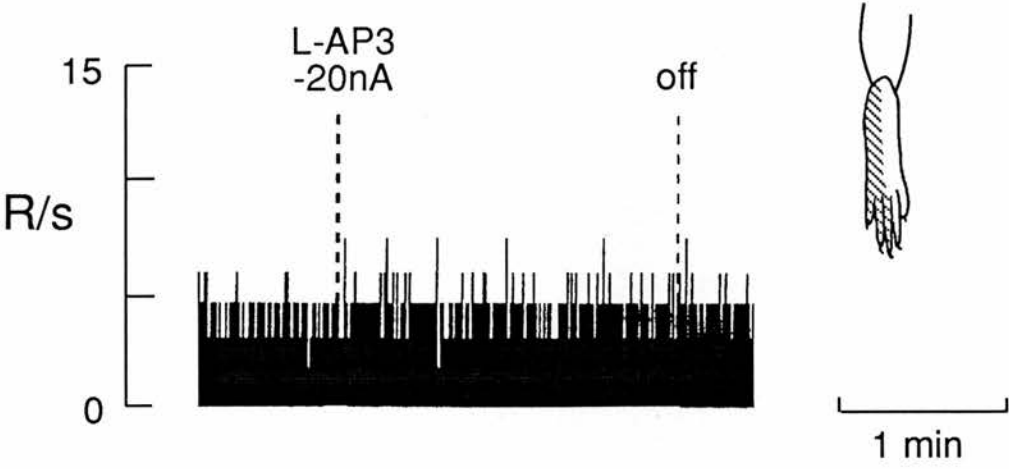
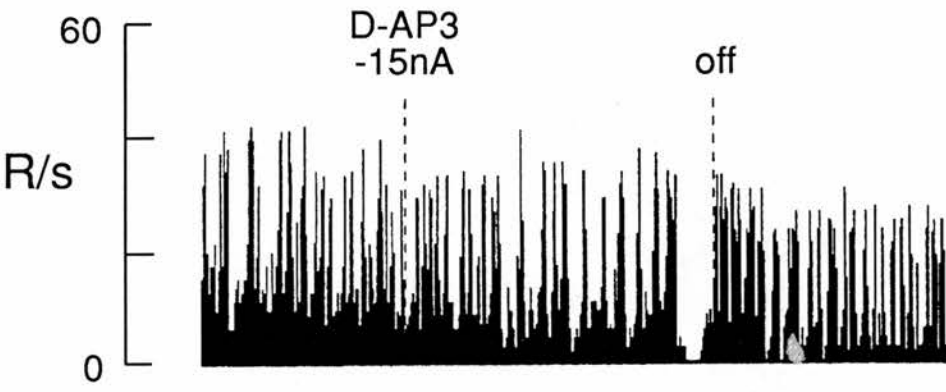
### **Figure 3.6**

#### **The effect of ionophoretically applied L- and D-AP3 on ACBD-evoked activity in dorsal horn neurons**

The records show ongoing firing of individual neurons, displayed as action potentials per second (R/s), integrated over 700 millisecond bins, plotted against time. The hatch areas on the insert shows the receptive field of the cell.

Firing was evoked by ionophoresis of the NMDA receptor agonist ACBD (-1nA), prior to and during the test. The selectivity of the AP3 isomers is demonstrated with D-AP3 showing an inhibitory effect, whereas the ejection of L-AP3, at an equivalent current, did not result in any change.

N.B. Due to the experimental set-up (manual operation of equipment) "off" was approximately 200msecs after ionophoresis of the drug ended.



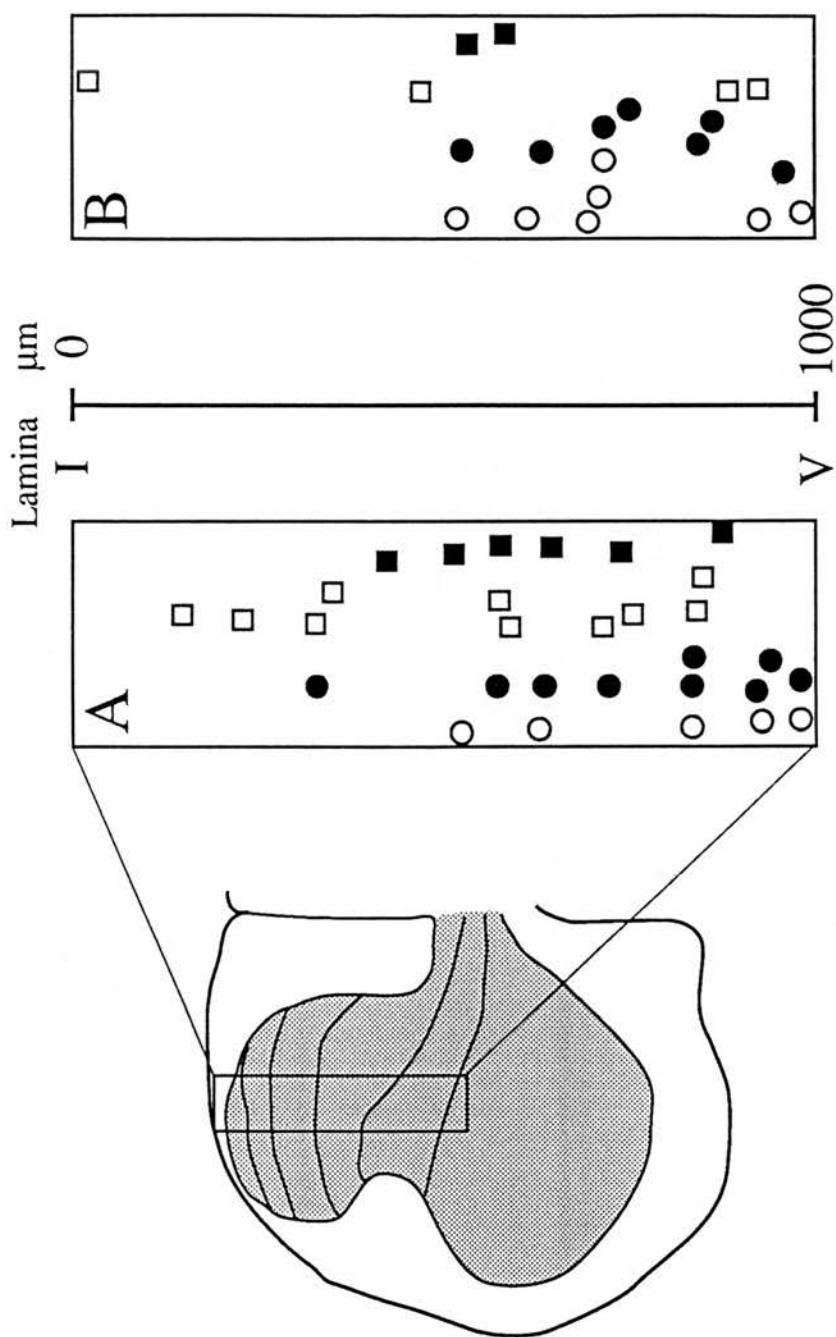
**Figure 3.7**

**Schematic representation of the relationship between neuronal responses to excitatory amino acid agonists, noxious heating of the cutaneous receptive field and microdrive depth readings**

The diagram represents neurons that did or did not respond to the ionophoretic application of either (A) metabotropic glutamate receptor agonist (1S,3R)-ACPD or (B) NMDA receptor agonist ACBD, and their response to noxious heating of their receptive field.

No correlation was found between the depth recorded by microdrive readings and the response to agonist ionophoresis. Similarly, there seemed to be no relationship between the response of a cell to agonist and heat.

- ✓ agonist, ✕ heat
- ✓ agonist, ✓ heat
- ✕ agonist, ✓ heat
- ✕ agonist, ✕ heat



can be seen in Figure 3.4. Recovery was observed in 15 of the 18 cells markedly inhibited by the antagonists. This occurred in under five minutes for all but 3 units, which required up to 15 minutes. As with the experiments on brush-evoked activity, neither of these compounds induced increased levels of firing. When the effect of (R,S)- $\alpha$ MeCPG was examined it had mixed effects. Four neurons in total were tested, 1 showed a clear inhibition whereas 2 showed a modest excitation ( $\leq 20\%$ ). Although this compound is a selective mGluR antagonist (Pook *et al*, 1993; Eaton *et al*, 1993) other such mixed effects on spinal motoneurons have recently been reported by Cao *et al* (1995).

### **3.3.4. EFFECTS OF MGLU RECEPTOR ANTAGONISTS ON NEURONAL RESPONSES TO EXCITATORY AMINO ACID AGONIST IONOPHORESIS**

In order to strengthen the evidence for the involvement of a genuine mGluR it was necessary to verify the selectivity of L-AP3 for mGluRs and D-AP3 for NMDA receptors under the present conditions. This was accomplished by testing both drugs on responses to agonists selective for each of these sites. (1S,3R)-ACPD was used to activate mGluRs. This compound acts best at mGluR subtypes 2 and 3, but also activates 1 and 5 (Tanabe *et al*, 1992; Tanabe *et al*, 1993; Watkins & Collingridge, 1994). ACBD is a highly selective NMDA receptor agonist (Lanthorn *et al*, 1990).

From background firing levels of 0-1 Hz, 14 out of 37 cells tested with (1S,3R)-ACPD (up to -80nA) responded with firing rates of 4-32 Hz. Sixteen out of 23 units responded to ACBD, to 4-40Hz. Agonists were ionophoresed at up to -80 and -90nA respectively, in order to see if a cell was responsive. However, cells that responded did so at much lower currents (-7 to -25nA for (1S,3R)-ACPD and -1 to -6nA for ACBD).

Agonist responses were tested comparatively with L- and D-AP3. In 4 out of 6 neurons activated by (1S,3R)-ACPD (-7 to -25nA) L-AP3 (-15 to -30nA) caused distinct inhibition of activity by  $67 \pm 12\%$  ( $p < 0.05$ , Wilcoxon test), whereas in all six cases D-AP3 had either no effect or caused a negligible excitation (see Fig 3.5). In contrast, D-AP3 (-15 to -30nA) reduced the firing elicited by ACBD (-1 to -6nA) in 4 out of 6 neurons by  $58 \pm 17\%$  ( $p < 0.05$ ), with L-AP3 failing to affect any (see Fig 3.6). In one cell both isomers were tested on each agonist and the expected selectivity was observed.

In a later study, (R,S)-CHPG (-15 to -40nA) was tested on (1S,3R)-ACPD-evoked activity in 5 neurons (see Fig 3.4). It caused a marked inhibition in all of them, by a mean of  $64 \pm 6\%$  ( $p < 0.05$ , Wilcoxon test). When the percentage inhibition values of brush-, mustard oil- and (1S,3R)-ACPD-evoked activity caused by (R,S)-CHPG were compared by the non-parametric Mann-Whitney U-test, effects on each of the other responses were greater than those on brush ( $p < 0.05$ ). (R,S)- $\alpha$  MeCPG was not assessed and (S)-CHPG was not available at the time of this study.

As a consequence of the lasting nature of activation caused by mustard oil, it was not possible to test drug effects in a side-by-side comparison if brush-, mustard oil- and (1S,3R)-ACPD-evoked responses on individual cells. Comparisons of a drug effect on brush/mustard oil, brush/(1S,3R)-ACPD or (1S,3R)-ACPD/mustard oil were however made in a number of cases. In individual examples of each combination both (R,S)-CHPG and L-AP3 caused a similar inhibition (by  $>50\%$ ) of mustard oil/(1S,3R)-ACPD response, but in examples of sequential brush/mustard oil and brush/(1S,3R)-ACPD tests the antagonists inhibited brush responses by  $<15\%$ , despite causing  $>50\%$  reduction in mustard oil and (1S,3R)-ACPD responses.

### 3.4. DISCUSSION

The population of neurons investigated in this study comprised of both multireceptive and non-nociceptive units, though all of the neurons used in the mustard oil study were multireceptive. The outcome of the pharmacological tests did not appear to bear any relation to the type of cell. On the basis of depth measurements made during the experiments along with histological examination of Pontamine Sky Blue ejection sites post-experimentally, the majority of cells were in laminae III-V of the dorsal horn of the spinal cord, as defined by Molander *et al* (1984). These neurons all exhibited very low basal firing rates of 0-1Hz. The routine ejection of NaCl vehicle at currents higher than those utilised for drug ejection caused no marked effects on any evoked activity throughout this study and failed to reproduce any of the drug-induced effects which were observed.

The mGluR agonist employed in this study, (1S,3R)-ACPD, was the only selective one widely available at the time. This compound, which caused excitation in under half of the cells tested (a higher proportion than those of Chapter 4, though the population was greater in that study) is a potent activator of group II mGluRs (Nakanishi, 1992; Tanabe *et al*, 1992; 1993), and is also an agonist at the group I



receptors (see Pin & Duvoisin, 1995). This data therefore suggests initially that the mGluRs involved here may be group II, or possibly group I. However, since this agonist-evoked activity was inhibited by L-(but not D-) AP3 and (R,S)-CHPG, both of which are antagonists at group I mGluRs, but not group II (Schoepp *et al*, 1990a; Watkins & Collingridge, 1994; Pin & Duvoisin, 1995), it would appear that the receptors in this instance are more likely to be group I. The data from the present study would suggest that the dorsal horn is not abundant in such (1S,3R)-ACPD-activated receptors. Comparing the data obtained in these experiments with that from the results from the NMDA receptor agonist, ACBD, which demonstrates that more than two-thirds of the population tested could be activated with this compound, there would appear to be a relatively sparse, though widespread (see Figure 3.7), distribution of (1S,3R)-ACPD-activated mGluRs in the spinal cord. The present study also demonstrates that neurons of the dorsal horn may contain both NMDA and mGlu receptors, as just over one quarter of the units tested could be activated by both agonists. However, neurons that could be excited by each compound individually were also observed, therefore there appear to be cells which display each receptor type exclusively (assuming that the ionophoresed drug was acting on the unit which was being recorded from). The ability of a neuron to respond to noxious heating of the receptive field did not reflect the presence or absence of any receptor type, mGlu or NMDA (see Figure 3.7). This would suggest that mGluRs are not exclusively involved in thermal nociception, but further studies are required to determine the possibility of their role in nociception of a mechanical nature, as no such monitoring of noxious pinch inputs was carried out in this instance.

The chemical irritant mustard oil (allyl isothiocyanate) is an activator of C-afferent nociceptive fibres (Woolf & Wall, 1986; Heapy *et al*, 1987) and its administration results in central sensitisation (Woolf & Wall, 1986). In this study the cutaneous application of mustard oil to the ipsilateral receptive field was used as an *in vivo* model of short-term but sustained activation of C-afferent fibres, in order to assess the role of mGluRs in nociception. Cleland *et al* (1994) demonstrated that mustard oil administered in this manner to spinalised, unanaesthetised rats resulted in a reproducible model of behavioural hyperalgesia. In this study such application of mustard oil (7.5% in paraffin oil) to the hairy (dorsal) surface of the skin led to an elevated firing rate (5-13Hz) of the neurons investigated, with these rises in background firing being maintained before any pharmacological testing was executed. Some neurons responded more readily to mustard oil, for unknown reasons, but the number of applications bore no apparent relevance on the outcome of pharmacological tests. An increase in the receptive field size often accompanied

such raised firing levels, a phenomenon which has been previously recorded with the use of this compound and is indicative of central sensitisation (Woolf & King, 1990; Woolf & Thompson, 1991). Other studies using mustard oil have found that it does not penetrate the skin well when applied to the glabrous surface (Harris & Ryall, 1988; F.E. Munro, PhD thesis, 1995), therefore the population of neurons investigated here were chosen according to their innervation of hairy skin. In a very small number of units (<5%) with thermal nociceptive inputs the topical administration of mustard oil failed to induce a sustained elevated firing rate, even following numerous applications (up to 7, over 35 minutes). This would suggest that there may be a sub-population of C-afferent fibres which, although they mediate noxious heat inputs, do not respond to this chemical. Thermo-specific receptors have been reported elsewhere (Davis *et al*, 1993) and may well exist in rat cutaneous structures. Alternatively it may be a simple case of the occasional inability of the chemical to reach the pertinent sites.

The experiments here provide evidence for a selective role of mGluRs in the processing of noxious chemical-, but not innocuous brush-evoked inputs. The firing induced by topical mustard oil could be markedly reduced when mGluR antagonists L-AP3 and (R,S)-CHPG were ionophoresed. Since D-AP3 brought about no change in any evoked firing, other than that elicited by ionophoresis of the NMDA agonist ACBD, it is highly likely that the effects of L-AP3 are indeed via an mGluR. At the time of these initial experiments (S)-CHPG was not available for testing, though it is likely that it too, as the isomer with greater activity as an mGluR antagonist (Glaum *et al*, 1993), would have resulted in a consistent decrease in this evoked activity. The mGluR antagonist (R,S)-CHPG used in these mustard oil experiments is a potent antagonist at the mGluR1, though shows little activity at the other subgroups of mGluRs (Watkins & Collingridge, 1994; Pin & Duvoisin, 1995). Although in general the properties of mGluR5 correspond broadly to those of mGluR1, some of the phenylglycine derivatives show some selectivity, as (S)-CHPG and (S)-4CPG have little affinity for mGluR5 (Joly *et al*, 1995). Although the (R)-enantiomer of CHPG displays some NMDA receptor antagonism (Watkins & Collingridge, 1994) it can be inferred that the inhibitory effects of (R,S)-CHPG are due to mGluR blockade since they are mimicked by L-AP3, and not its isomer D-AP3 (also a weak NMDA antagonist; Schoepp *et al*, 1990a). L-AP3 and (R,S)-CHPG along with the mGluR selective isomer, (S)-CHPG failed to alter the firing elicited by light innocuous brushing of the receptive field. Statistical comparisons revealed that the inhibitory effect of these antagonists on agonist/mustard oil-evoked activity was significantly greater than the effect on brush-elicited activity. It would therefore seem apparent

that mGluRs, probably subtypes 1 and/or 5 are involved in the mediation of nociceptive information via C-, and perhaps A $\delta$ -, afferent fibres though not low threshold (A $\beta$ ) inputs. The mixed effects observed with  $\alpha$ -MeCPG in the initial mustard oil study are not unique. Cao *et al* (1995) recently observed similar dual effects with this compound, a reported weak antagonist at several mGluRs (Eaton *et al*, 1993; Pook *et al*, 1993; Pin & Duvoisin, 1995), in a study on spinal motoneurons. Nevertheless, because of its apparently inconsistent effects at the time of these pilot experiments it was decided that  $\alpha$ -MeCPG would not be used in further investigations.

The evidence presented here for a discrete role for mGluRs is in agreement with several other studies which have also demonstrated such selectivity in sensory processing. In thalamic nuclei, Eaton *et al* (1993a; 1993b) found that locally administered (S)-CHPG reduced firing elicited by noxious thermal, but not innocuous air-jet stimulation. Using newly available selective compounds this group have recently provided evidence that pre- and post-synaptic (group II/III and group I respectively) mGluRs exist in the ventrobasal thalamus and may play a role in the disinhibition of various sensory (and electrically-evoked) inputs (Salt & Eaton, 1995a; 1995b). In addition to these studies, Neugebauer *et al* (1994b) showed how L-AP3 could only reduce the responses of spinal neurons to pressure applied to the knee after the induction of inflammation, and not before. Taken together with the present study, these results indicate that mGluRs are effective only in the transmission of high threshold sensory inputs, with particular relevance in a sensitised state. There are however studies which failed to find evidence for such a role of these receptors. Thompson *et al* (1992) found that L-AP3 did not affect ventral root potentials evoked by high intensity (C-fibre) stimulation *in vitro*, this could of course have been due to the fact that the preparation was a neonatal rat spinal cord, and the potency of L-AP3 at mGluRs is lower in neonatal tissue than in adult (Schoepp & Johnson, 1989a; 1989b). Also, the difference in stimulus type and population of neurons tested are likely to contribute to a variance in outcome. An *in vivo* investigation by Palecek *et al* (1994) found mixed effects using (1S,3R)-ACPD. They observed an increase in the response of primate STT cells to innocuous brushing of the receptive field when a low dose of the agonist was applied by microdialysis, yet no change in responsiveness with a higher dose. Neither dosage affected responses to noxious mechanical or thermal stimulation. This anomaly with the present results is not easily explicable. However, there are several considerations: firstly it must be remembered that data from experiments using agonists should not necessarily be directly compared to that obtained with

antagonists, as the physiological consequences need not necessarily be opposites. Secondly (1S,3R)-ACPD does not activate solely the exact complement of mGluRs which are antagonised by the compounds used in the present study. Finally, the distance from the recorded neurons and the ability of the compound to reach that neuron adequately may well vary in different studies.

The mechanism by which mGluRs mediate their effects is unknown, though changes in excitability brought about by channel regulation may be one of the mechanisms involved. The modulation of  $\text{Ca}^{++}$  and  $\text{K}^+$  channels, both inhibition and activation, by group I mGluRs (and to a much lesser extent group II/III) has been observed, and there is evidence that this may be mediated by second messengers such as PKC (Baskys, 1992; Swartz & Bean, 1992; Swartz *et al*, 1993; Chavis *et al*, 1994; see also Pin & Duvoisin, 1995). Additionally, there is interesting evidence that mGluRs may act through a more specific functional interaction with NMDA, and perhaps other, receptors. Potential facilitation of NMDA receptor function is of special interest for 3 reasons:

(i) it is strongly implicated in the sensitisation of dorsal horn neurons during repetitive stimulation (Davies & Lodge, 1987; Dickenson & Sullivan, 1987; Dickenson & Sullivan, 1990; Thompson *et al*, 1990),

(ii) mGluR (group I) facilitation of NMDA receptor function is likely to be an important component of at least some forms of LTP in the hippocampus (a phenomenon with many functional analogies to dorsal horn neuron sensitisation) and

(iii) molecular mechanisms (PKC phosphorylation of the NMDA receptor) have been described (Chen & Huang, 1992; Tingley *et al*, 1993) which could explain how a phospholipase C-coupled receptor (such as a group I mGluR) could enhance NMDA receptor function.

Early *in vitro* studies showed how (1S,3R)-ACPD could potentiate ionotropic glutamate receptor agonist-induced responses in isolated dorsal horn neurons and a spinal cord slice preparation (Bleakman *et al*, 1992; Cerne & Randic, 1992). The use of more specific agonists by Bond & Lodge (1995) recently confirmed this *in vivo*, with the suggestion of Group I or II mGluRs playing the crucial role. Interestingly, Jones & Headley (1995) concluded from a similar study that such elevated NMDA-evoked activity was not due to a potentiation by the mGluR agonist, but instead a simple additive rise in background activity. Conceivably mGluRs may be involved in a gating mechanism whereby their initial activation allows the triggering of other receptor activity, in this case the NMDA receptor (but perhaps also other receptors), which would otherwise require a synaptic input differing in intensity, modality or perhaps temporal requirements. Other more



general changes in excitability may well occur, perhaps with more intense or prolonged activation of mGluRs or perhaps concurrently with a more specific regulation of NMDA receptor function. In CA1 hippocampal neurons mGluR antagonists can block an initial LTP without affecting later trials, and on this basis it has been suggested that prolonged molecular events are induced - thus the persistent activation of mGluRs is not necessary beyond the primary 'switch' event (Bortolotto *et al*, 1994). The delivery of a low-frequency electrical stimulus in this LTP study reversed the mGluR 'switch', so the changes do not appear to be irreversible and the state of responsiveness seems to be dependant on input frequency. This may also be the case in the transmission of analogous sustained nociceptive inputs, but obviously further experiments are required to ascertain the exact nature of mGlu activation and the consequences of this. Since the present study did observe an inhibition of mustard oil-evoked activity with mGluR antagonists it is unlikely that the mechanisms are identical. However, it would be prudent to now test the effect of mGluR antagonists on a brief noxious input to dorsal horn neurons both before and after mustard oil application, in order to assess the role of the antagonists pre- and post-sensitisation, and to test the specificity of mGluR subtypes in both instances.

As well as the possible physiological mechanisms by which mGluRs may act, their pharmacology is becoming increasingly complex. Certain compounds, including some of the available phenylglycine derivatives, appear to act preferentially with receptor subgroups, but there is often a degree of overlap thus making it difficult as yet to fully characterise the receptors studied *in vivo*. L-AP3 is a weak group I mGluR antagonist while (R,S)- and (S)-CHPG are potent antagonists at these receptors, particularly mGluR1 (Watkins & Collingridge, 1994; Pin & Duvoisin, 1995). It would therefore seem appropriate to deem the receptors involved in this model of sustained nociception as either mGluR1 and/or mGluR5. However, the lack of entirely specific antagonists available at the time of these experiments makes further judgement unfounded. Recent studies in our laboratory using 3,5-DHPG, a selective group I agonist and cyclothiazide (CTZ) an mGluR1 selective antagonist ineffective at mGluR5 (Sharp *et al*, 1994), have provided some evidence demonstrating the specific presence of the mGluR1 receptor in the dorsal horn and its involvement in mustard oil-evoked activity (S. Fleetwood-Walker & H. Sparrow, unpublished observations). Ideally the experiments in this study could be repeated using such newly available selective mGluR agonists which allow distinction between the subtypes in question. Selective group II and group III agonists e.g. CCG-I and L-AP4 respectively (Hayashi *et al*, 1992; Thomsen *et al*, 1992; Nakajima *et al*, 1993; Tanabe *et al*, 1993; Okamoto *et al*, 1994; Duvoisin *et al*, 1995), would

also be of interest since, although the mGluRs involved in the present experiments appear to be of group I, it seems likely that other mGluRs will exert relevant influences in sensory processing in the dorsal horn. The ongoing development of increasingly selective antagonists will be crucial in providing unequivocal evidence as to the nature of the receptors which mediate the C fibre inputs and central sensitisation generated by the cutaneous application of mustard oil, as well as other potential mGluR-mediated modulation of dorsal horn neuron function.

## CHAPTER 4:

### STUDIES ON THE ROLE OF DIFFERENT INTRACELLULAR SIGNALLING PATHWAYS IN RESPONSES OF DORSAL HORN NEURONS TO SENSORY STIMULI AND TO A METABOTROPIC GLUTAMATE RECEPTOR AGONIST

#### 4.1 AIMS

The effect of inhibitors of several second messenger systems on innocuous brush- and noxious chemical (mustard oil)-evoked activity was examined, to elucidate any specific pathways involved in nociception.

Secondly, in order to investigate possible intracellular signalling pathways linked to metabotropic glutamate receptors (mGluRs), these inhibitors were ionophoresed, whilst the cell was activated with an mGlu agonist.

#### 4.2. METHODS

##### 4.2.1. General Methods

Regarding animals, surgical procedures and electrophysiological recording techniques, quantification of neuronal responses to evoked activity, histological identification of recording sites and analysis of results please refer to section 3.2.

##### 4.2.2. Drugs for Ionophoresis

All solutions were pH 4.0-4.5. Drugs were dissolved at the greatest concentration compatible with an acceptable concentration of vehicle,  $\leq 0.5\%$  dimethylformamide (DMF) in the final electrode solution.

*Calmodulin/ $Ca^{++}$ -Dependent Kinase II:* calmidazolium (Silver *et al*, 1986) and KN62 (Tokumitsu *et al*, 1990) were both  $50\mu\text{M}$  in  $0.5\%$  DMF.

*Protein Kinase C:* GF109203X (Toullec *et al*, 1991) was  $100\mu\text{M}$  in  $0.5\%$  DMF and chelerythrine (Herbert *et al*, 1990) was  $1\text{mM}$  aqueous.

*Phospholipase  $A_2$ :* ONO-RS-082 (Banga *et al*, 1986) was  $100\mu\text{M}$  in  $0.5\%$  DMF and benzenesulphonamide 4 (Oinuma *et al*, 1991)  $50\mu\text{M}$  in  $0.5\%$  DMF.

*Protein Kinase A:* KT5720 (Kase *et al*, 1987) was  $50\mu\text{M}$  in  $0.5\%$  DMF and H89 (Chijiwa *et al*, 1990)  $100\mu\text{M}$  in  $0.5\%$  DMF.

*Tyrosine Kinase*: lavendustin A (Hsu *et al*, 1991) was 200 $\mu$ M in 0.2% DMF and piceatannol (Geahlen *et al*, 1989) 300 $\mu$ M in 0.3% DMF.

#### **4.2.3. Controls**

As well as 1M NaCl, 0.5% DMF was ionophoresed up to 80nA for simultaneous current and vehicle controls. Both controls were carried out on agonist-, brush- and mustard oil-evoked activity.

#### **4.2.4. Analysis of Results**

For details on analysis please refer to section 3.2.10. Note that changes brought about in neuronal firing which were within 15-20% of pre-drug controls were not considered to be marked, as they were within natural variability brought about by vehicle or current controls (although these were normally <10%).

### **4.3 RESULTS**

#### **4.3.1.1. Characteristics of Neurons**

A total of 43 rats were used for this study, with the investigation of 169 neurons. Microelectrode depths ranged from 150-900 $\mu$ m, from the surface of the spinal cord. Resting discharge was between 0 and 1Hz. Most neurons in this study were found to have receptive fields on the cutaneous surface of the ipsilateral hindlimb. The size of the receptive field was variable, ranging from a single digit to the majority of the upper leg, although field size did not appear to result in any differences observed with the drugs used.

In the overall population the characteristics of neurons which were or were not inhibited by antagonist ionophoresis did not differ. Seventeen cells were tested sequentially with several drugs, on either responses to brush (6 cells), mustard oil (6 cells) or agonist (5 cells). No differences in results were seen due to the order of application of drugs. Time for full recovery was allowed between these sequential tests (up to 15 minutes) and the results from such multiple trials were always typical of the overall population. All evoked activity was always maintained at steady levels throughout testing, from 10 to 40 minutes.

In a total of 76 neurons, innocuous brushing of the receptive field resulted in a rise from basal discharges of 0-1Hz to steady firing rates between 10 and 40Hz. In the mustard oil study 38 multireceptive cells were used. All of these neurons were required to give a vigorous response to noxious heating of the



cutaneous receptive field. Repeated topical applications of mustard oil to the receptive field of these neurons produced elevated levels of firing from 4-37Hz. Metabotropic glutamate receptor agonist, (1S,3R)-ACPD, was ejected between 0 and -10nA, and produced steady firing rates from 7-50Hz. Approximately one fifth (47 out of 244) of neurons were excited by such ionophoresis of the agonist. On only 2 occasions in the 61 tests carried out did an inhibitor fail to reduce agonist-evoked activity, so it is possible that all five of the intracellular signalling pathways investigated here are linked to at least one of the four metabotropic glutamate receptors (mGluRs 1, 2, 3 and 5) activated by this compound. Alternatively, a number of the compounds may have secondary effects additional to their main influence on signalling pathways. The degree of inhibition on (1S,3R)-ACPD-evoked activity was similar for all compounds.

Recovery was observed in approximately three quarters of the tests, ranging from a few seconds after the termination of drug ionophoresis to 10-15 minutes later. No particular compounds allowed recovery on more occasions than the others.

#### **4.3.1.2. Ionophoretic and Vehicle Controls**

As well as the control data presented in section 3.3.1.2., it was necessary to test the effect of ionophoresed vehicle containing dimethylformamide (DMF), as some of the compounds were prepared in that vehicle. 0.5% DMF was ejected at up to +80nA for 2-5 minutes during brush-, mustard oil- and agonist-evoked firing. It failed to markedly alter activity (never beyond 15% of pre-drug controls;  $p > 0.05$ , Wilcoxon test) in 6 out of 6, 4 out of 4 and 4 out of 5 cases respectively (see Fig 4.1). On the one occasion where it did reduce (1S,3R)-ACPD-induced activity, it did so at +80nA. This high level of ejection current was only ever employed for the ionophoresis of the protein kinase C inhibitor chelerythrine, and the effects of this compound could not be due to DMF as it was dissolved in water.

#### **4.3.2. EFFECTS OF INHIBITORS OF INTRACELLULAR SIGNALLING PATHWAYS ON NEURONAL RESPONSES TO INNOCUOUS BRUSH STIMULI**

##### **4.3.2 (a) Calmodulin/ $\text{Ca}^{++}$ -dependent kinase II Inhibitors (Calmidazolium and KN62)**

Twenty neurons which had a response to innocuous brush were studied. Of the 8 units in which an antagonist caused an inhibition beyond control margins

(15-20% of pre-drug levels), all responded to noxious pinch and 1 of them to noxious heating of the cutaneous receptive field. These compounds were predominantly ineffective in altering brush-evoked activity (see Figs 4.2a and 4.2b). Calmidazolium (30-60nA) caused an overall mean decrease of  $16 \pm 10\%$  from pre-drug controls ( $n=8$ ), this effect was not statistically significant ( $p>0.05$ , Wilcoxon test). Two of the 8 units were inhibited beyond the margin of variability (by 69% and 49%). Of the 6 cells which were not inhibited more than 15-20%, 3 showed negligible alterations in firing during drug iontophoresis, 2 showed small inhibitions (16 and 18%) and 1 had its firing slightly facilitated (+11% from control levels). KN62 (30-70nA) did cause a significant inhibition in the overall population of neurons ( $p<0.05$ , Wilcoxon test), with a mean decrease in activity of  $42 \pm 13\%$ . However, KN62 only markedly reduced firing ( $>15-20\%$ ) in 6 out of 12 tested neurons. Of the remaining 6 cells, 2 showed no measurable change, 3 were inhibited less than 15-20% and 1 showed a small facilitatory effect of the compound (+26%).

#### **4.3.2 (b) Protein Kinase C (PKC) Inhibitor (GF109203X)**

The effect of GF109203X on firing induced by brushing of the receptive field was investigated by Munro *et al* (1994a). This study showed that only 1 of 5 tested units was inhibited beyond 15-20% by this compound, with a mean inhibition of  $3 \pm 3\%$  for the whole group; not significantly different from pre-drug control values ( $p>0.05$ , Wilcoxon test).

#### **4.3.2 (c) Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) Inhibitors (ONO-RS-082 and BS4)**

A total of 18 neurons were investigated, 9 of which had their brush-evoked activity significantly inhibited by the PLA<sub>2</sub> antagonists. Seven of these neurons were tested to noxious pinch, 6 responded. One cell was non-nociceptive, 2 were not characterised. These compounds were slightly more effective in reducing activity evoked by brushing of the receptive field than the PKC inhibitors, but nevertheless they only considerably inhibited half of the total number of cells tested (see Figs 4.4a and 4.4b for typical examples of non-inhibition). ONO-RS-082 (30-65nA) caused a mean inhibition of  $28 \pm 13\%$  ( $n=9$ ), a statistically insignificant effect ( $p>0.05$ , Wilcoxon test). Of the 9 units which were investigated, 4 had their activity reduced beyond 15-20% and the remaining 5 showed decreases in activity from 3-6%. In the population of cells tested with BS4 (30-60nA) a significant lowering of evoked activity occurred overall ( $-37 \pm 12\%$  from control values;  $p<0.05$ , Wilcoxon test). However, only 5 of 9 cases tested were reduced beyond the 15-20% margin of

variability. Of the 4 which did not have markedly reduced firing, 2 showed a slight decrease in firing (by 13 and 19%), 1 showed no detectable change, and the remaining cell was excited by 8%.

#### **4.3.2 (d) Protein Kinase A (PKA) Inhibitors (KT5720 and H89)**

Twenty neurons were examined. Of the 12 which were showed a reduction in brush-evoked activity due to these compounds all but 1 was tested, and fired in response, to noxious pinching. One cell was also activated by noxious heat. Ionophoretic application of these compounds resulted in a lowered firing rate in just over half of the neurons tested (see Figs 4.5a and 4.5b for examples of inhibition). Both drug-induced effects were statistically significant ( $p < 0.05$ , Wilcoxon test). KT5720 (20-60nA) reduced neuronal firing by a mean of  $59 \pm 15\%$  across the population of cells tested ( $n=10$ ), although 3 units showed less than 15-20% change from pre-drug control values. Two of these 3 cells showed no measurable change and 1 had its firing decreased by 8%. Ionophoresis of H89 (30-70nA) resulted in a mean decrease across the population of  $45 \pm 15\%$  ( $n=10$ ), although only 5 cells were inhibited beyond the error margin of 15-20%. The remaining 5 neurons did not have their activity altered by more than 10% from pre-drug control values.

#### **4.3.2 (e) Tyrosine Kinase Inhibitors (Lavendustin A and Piceatannol)**

A total of 24 neurons were studied. Of the 16 in which the inhibitors were effective on brush-evoked activity, 9 responded to noxious pinching and 1 also responded to noxious heating of the cutaneous receptive field. Four cells were non-nociceptive and 3 were uncharacterised. Two thirds of the units tested here were markedly inhibited by these compounds. Lavendustin A (30-70nA) caused a mean decrease of  $60 \pm 12\%$  across the population ( $n=11$ ;  $p < 0.05$ , Wilcoxon test), reducing brush-evoked activity beyond the 15-20% error margin in 9 out of 11 cases, with the other 2 neurons showing no detectable change. Piceatannol (7-50nA) inhibited activity by a mean of  $48 \pm 15\%$  ( $n=13$ ;  $p < 0.05$ , Wilcoxon test). This reduction was greater than the margin of variability in 7 of the 13 units. Three of the remaining 6 cells showed no noticeable change in activity from pre-drug levels, and in the other 3 units activity was slightly elevated (+8, 16 and 20% from control levels). See Figs 4.6a and 4.6b.

### **Figure 4.1**

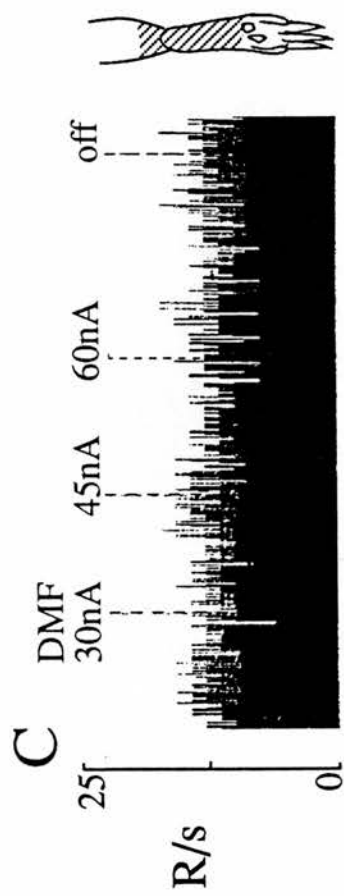
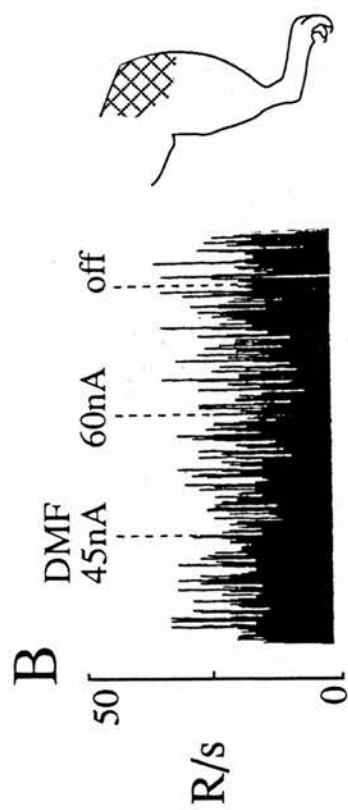
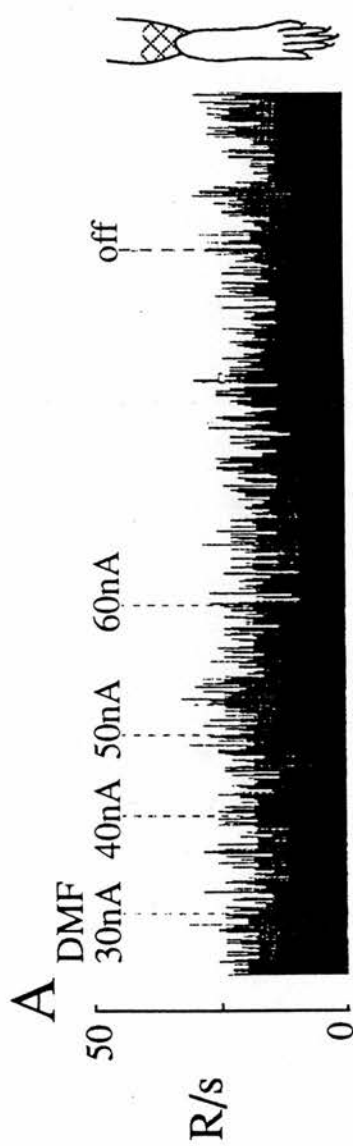
#### **Lack of effect of 0.5% dimethylformamide on evoked activity in dorsal horn neurons**

The records show ongoing firing of individual neurons, displayed as action potentials per second (R/s), integrated over 700 millisecond bins, plotted against time.

As many of the second messenger inhibitors were dissolved in up to 0.5% dimethylformamide (DMF), ionophoresis of this vehicle during evoked responses was investigated.

The records show how ejecting 0.5% DMF at up to 60nA (up to 70nA in other cases) failed to alter firing evoked by (A) innocuous brush, (B) mustard oil and (C) (1S, 3R)-ACPD.

The inhibitory effect of ionophoresis of calmodulin/ $\text{Ca}^{++}$ -dependent kinase II inhibitor KN62 can be seen on the same neuron shown in record (B), in Figure 4.2b (B).



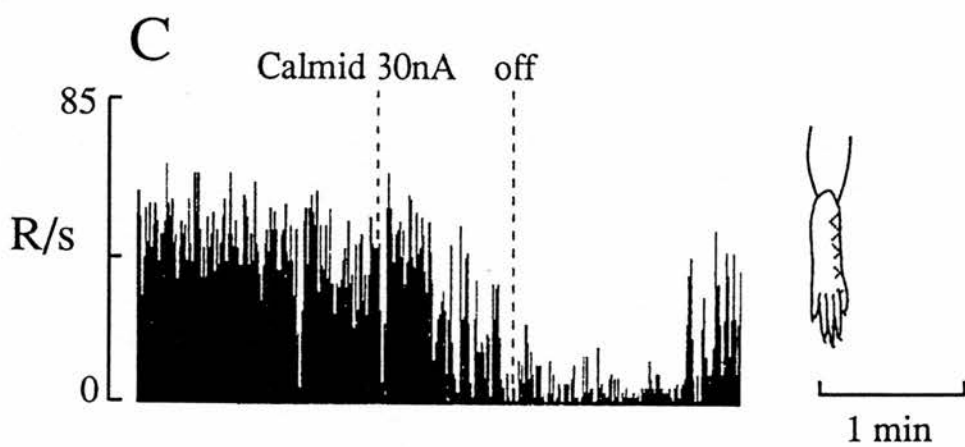
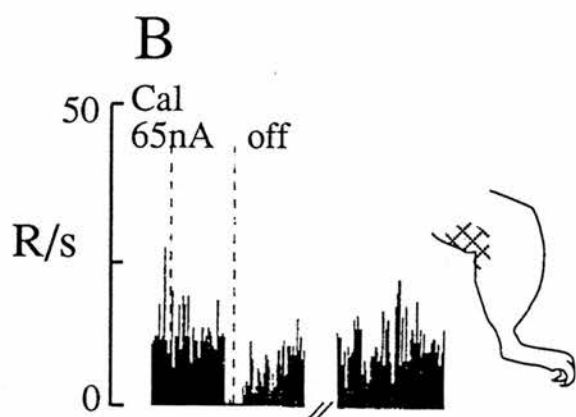
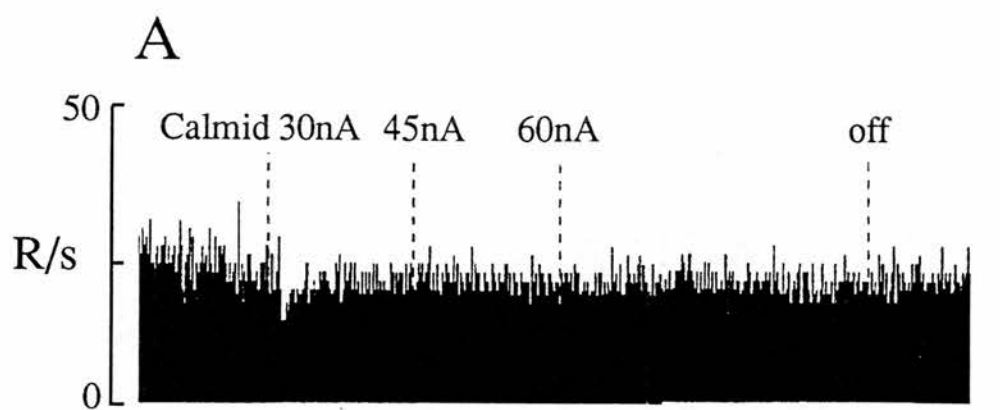
1 min

### **Figure 4.2a**

#### **The effect of ionophoretically applied Calmidazolium, a calmodulin/ $\text{Ca}^{++}$ -dependent kinase II inhibitor, on evoked activity in dorsal horn neurons**

The records show ongoing firing of individual neurons, displayed as action potentials per second (R/s), integrated over 700 millisecond bins, plotted against time. The inserts show receptive fields of cells, with hatched areas representing both sides of the leg/foot.

These are typical examples of how calmidazolium affected evoked responses, and the similar effects of KN62 are shown in Figure 4.2b. In most cases calmidazolium did not alter the firing evoked by innocuous brushing of the cutaneous receptive field, as in (A). However, it did decrease activity induced by both mustard oil (B) and the metabotropic glutamate receptor agonist (1S,3R)-ACPD (C).



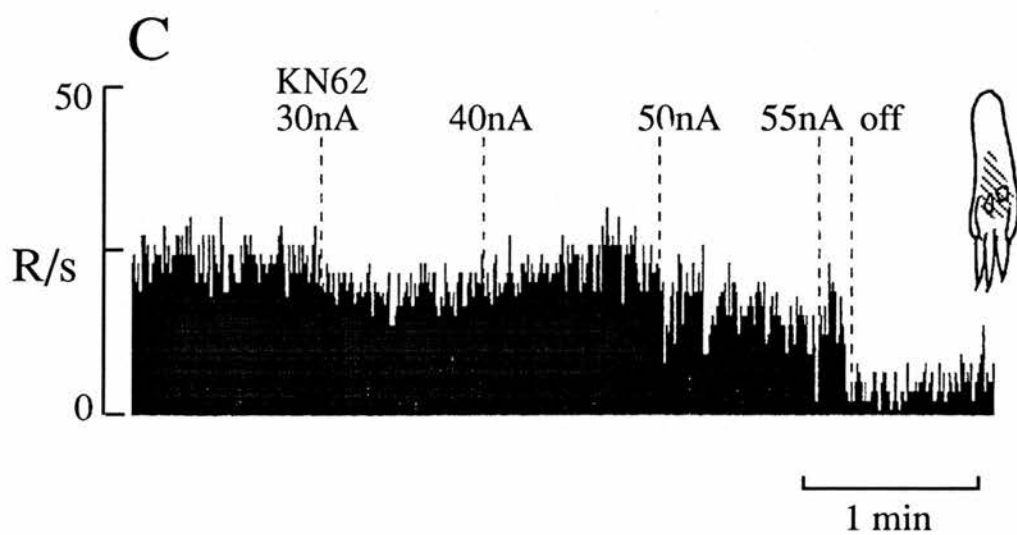
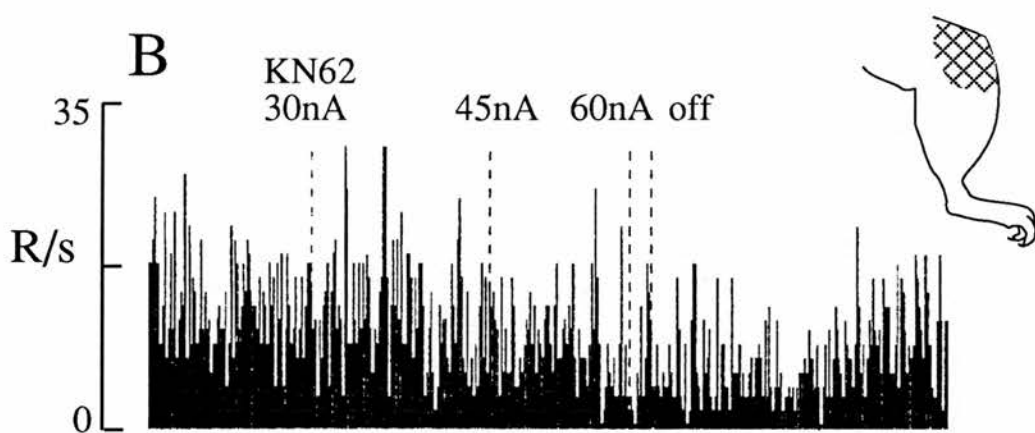
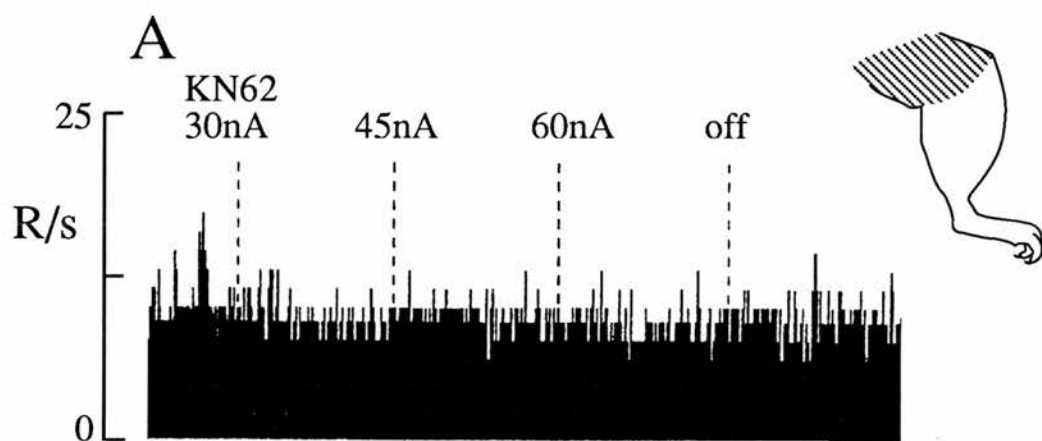
### **Figure 4.2b**

**The effect of ionophoretically applied KN62, a calmodulin/ $\text{Ca}^{++}$ -dependent kinase inhibitor, on evoked activity in dorsal horn neurons**

The records show ongoing firing of individual neurons, displayed as action potentials per second (R/s), integrated over 700 millisecond bins, plotted against time.

These are typical examples of how KN62 affected evoked responses. In most cases KN62 did not alter the firing evoked by innocuous brushing of the cutaneous receptive field, as in (A). However, it did decrease activity induced by both mustard oil (B) and the metabotropic glutamate receptor agonist (1S,3R)-ACPD (C).



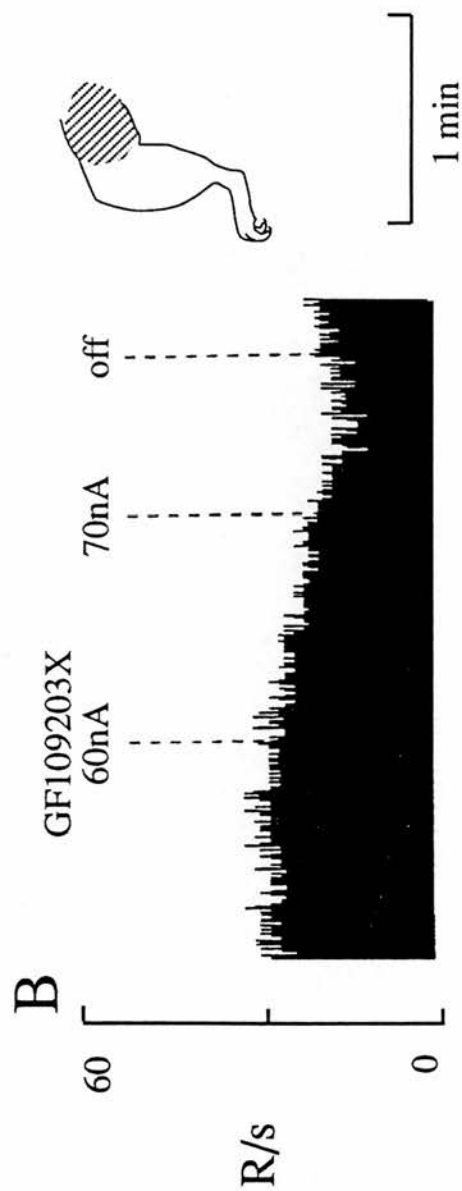
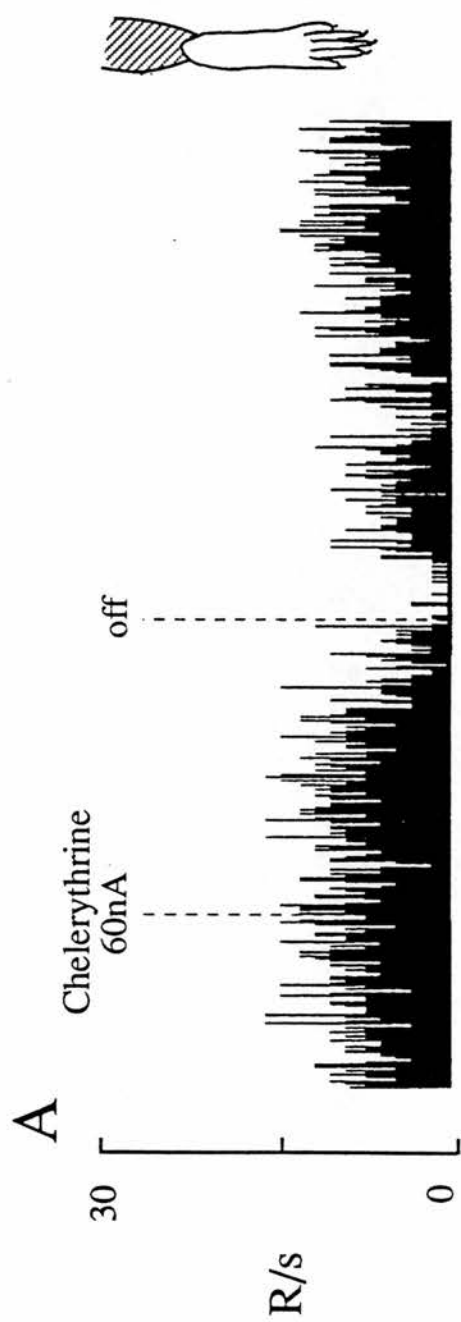


### Figure 4.3

**The effect of ionophoretically applied chelerythrine and GF109203X, protein kinase C inhibitors, on (1S,3R)-ACPD evoked activity in dorsal horn neurons**

The records show ongoing firing of individual neurons, displayed as action potentials per second, integrated over 700 millisecond bins (R/s), plotted against time. Receptive fields are shown by diagonal lines on the inserts.

Firing was evoked by ionophoresis of metabotropic glutamate receptor agonist (1S,3R)-ACPD (1nA in (A) and 3nA in (B)) prior to and during the test. The records show typical examples of how both protein kinase C inhibitors reduced such activity.

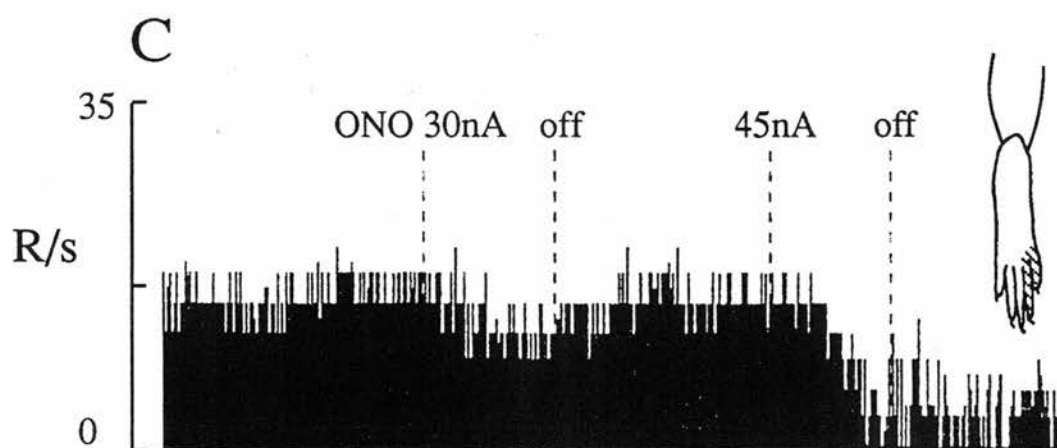
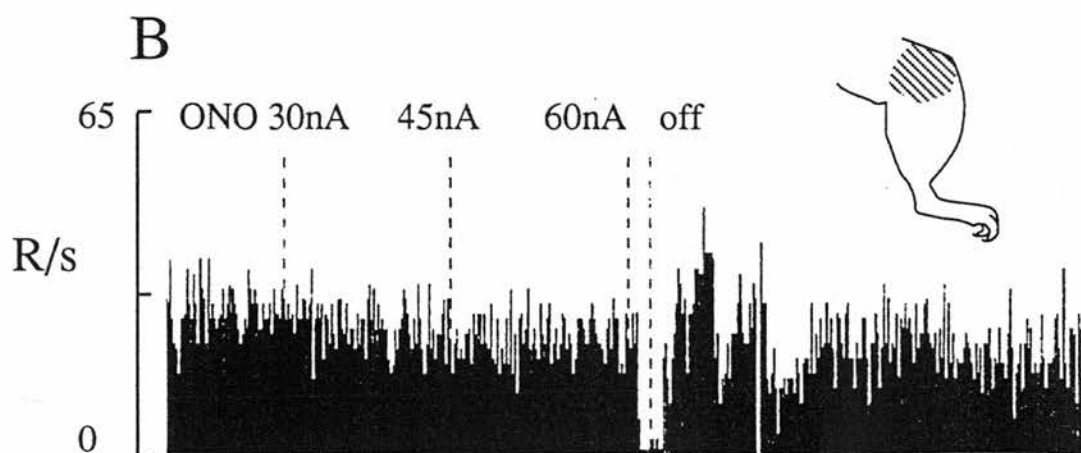
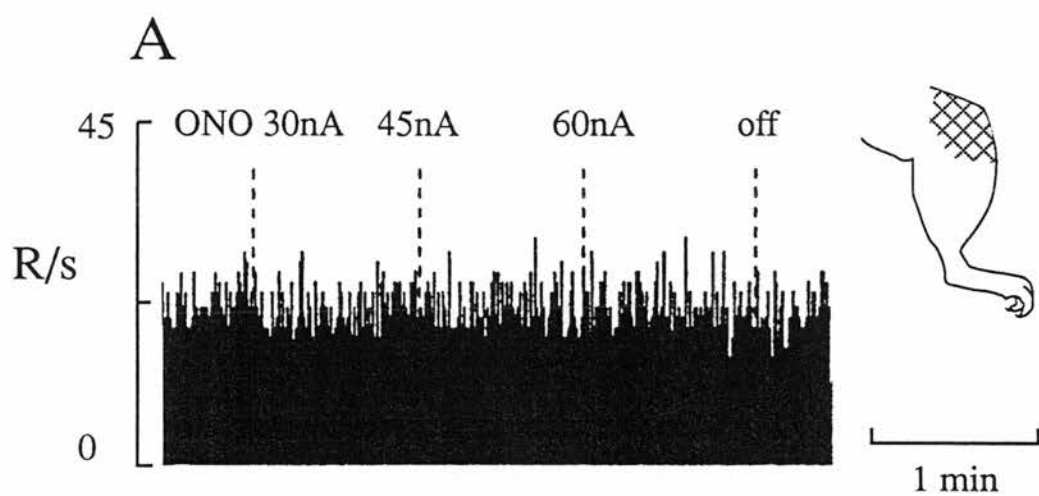


### **Figure 4.4a**

#### **The effect of ionophoretically applied ONO-RS-082, a phospholipase A<sub>2</sub> inhibitor, on evoked activity in dorsal horn neurons**

The records show ongoing firing of individual neurons, displayed as action potentials per second (R/s), integrated over 700 millisecond bins, plotted against time. Diagonal lines on the inserts show the receptive field of the cells, with hatched areas representing both sides of the leg/foot.

The lack of effect of ONO-RS-082 on brush-evoked activity (A) can be contrasted with its inhibitory effects on both (B) mustard oil- and (C) (1S,3R)-ACPD (-7nA) -evoked firing. At equivalent currents, this compound can be clearly seen to reduce a noxious input to a greater extent than an innocuous input.



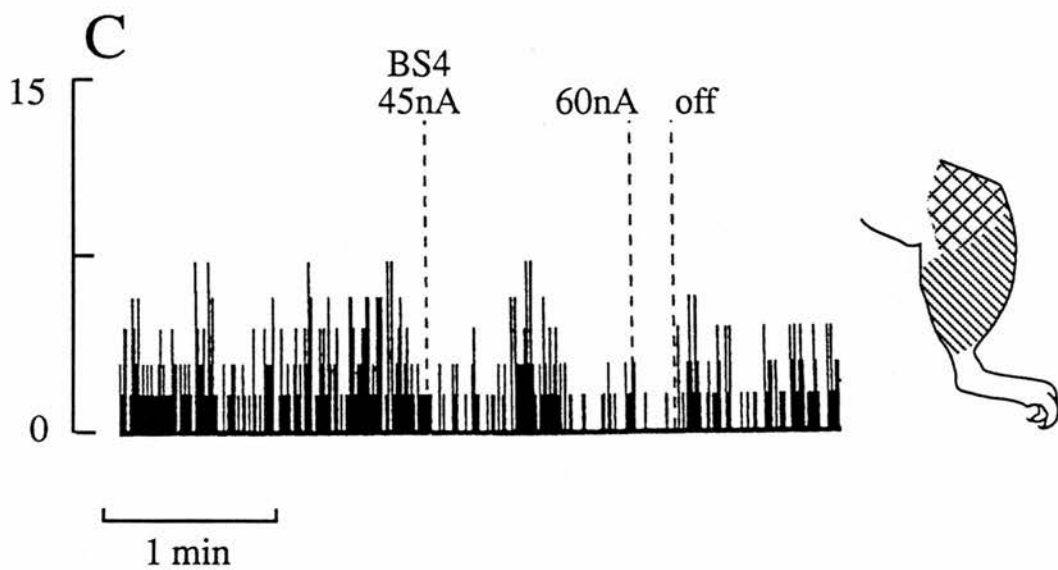
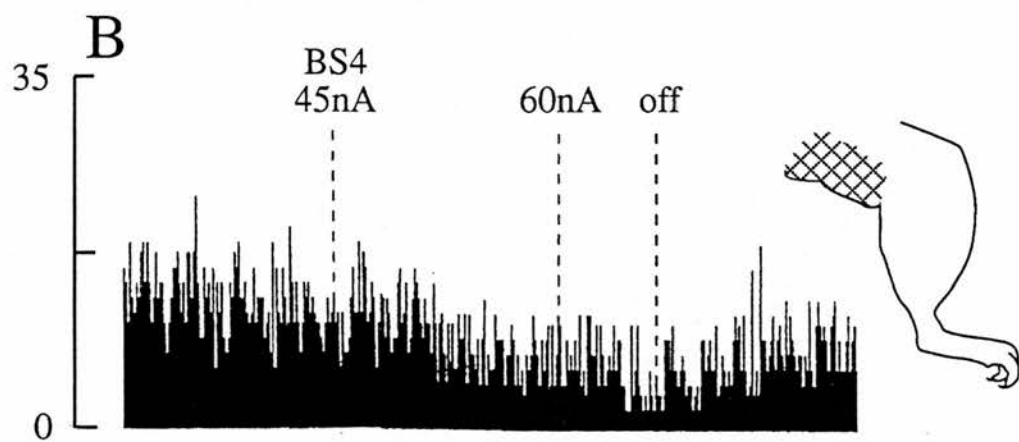
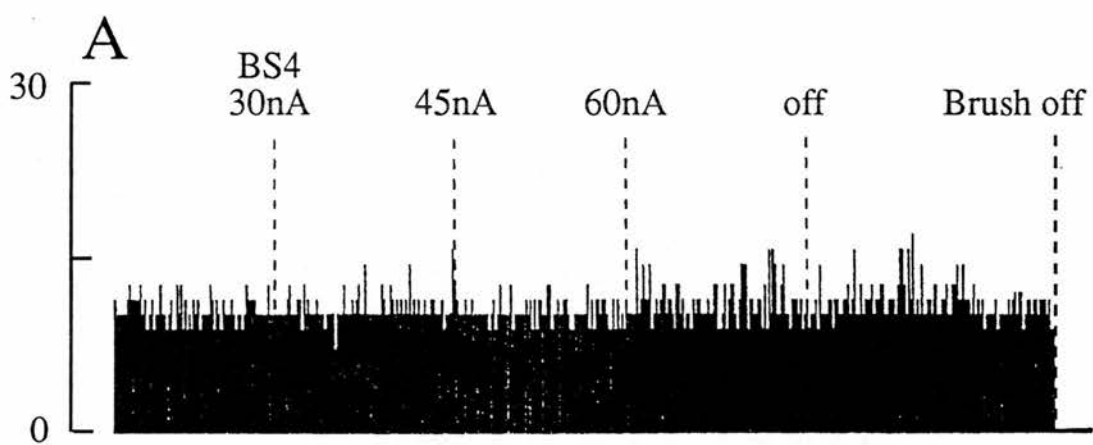
### Figure 4.4b

#### **The effect of ionophoretically applied Benzenesulphonamide (BS4), a phospholipase A<sub>2</sub> inhibitor, on evoked activity in dorsal horn neurons**

Ionophoresis of a second phospholipase A<sub>2</sub> inhibitor, BS4, resulted in the same overall effect. The records show ongoing firing of individual neurons, displayed as action potentials per second (R/s), integrated over 700 millisecond bins, plotted against time.

The lack of effect of BS4 on brush-evoked activity (A) can be contrasted with its inhibitory effects on both (B) mustard oil- and (C) (1S,3R)-ACPD (-5nA) -evoked firing. At equivalent currents, this compound can be clearly seen to reduce a noxious input to a greater extent than an innocuous input.

Traces A and C are from the same neuron (common receptive field alongside C), demonstrating how the compound selectively reduced agonist-evoked activity, but not that of brush.



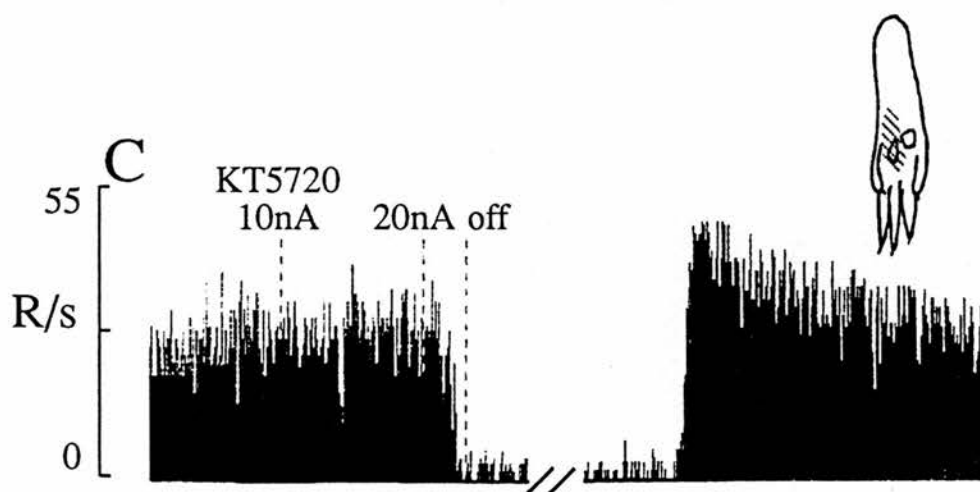
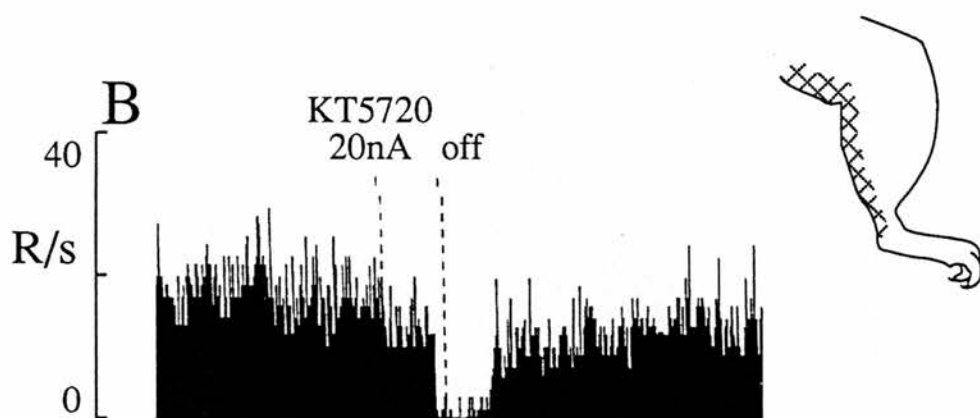
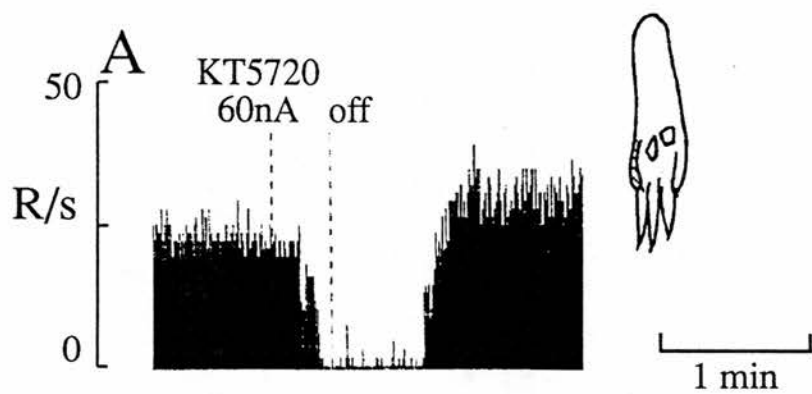
### Figure 4.5a

#### **The effect of ionophoretically applied KT5720, a protein kinase A inhibitor, on evoked activity in dorsal horn neurons**

The records show ongoing firing of individual neurons, displayed as action potentials per second, integrated over 700 millisecond bins (R/s), plotted against time. The receptive field is shown on the inserts by diagonal lines, or hatched areas where it occurred on both sides of the leg.

Record (A) is an example of how ionophoresis of KT5720 led to a reduction in brush-evoked firing. This was typical in over half of the neurons tested. Traces (B) and (C) demonstrate how, at much lower currents, KT5720 also reduced the firing evoked by the cutaneous application of mustard oil and ionophoretic application of metabotropic glutamate receptor agonist (1S,3R)-ACPD (-1nA). The broken lines in (C) represent 4 minutes which passed before recovery was observed.



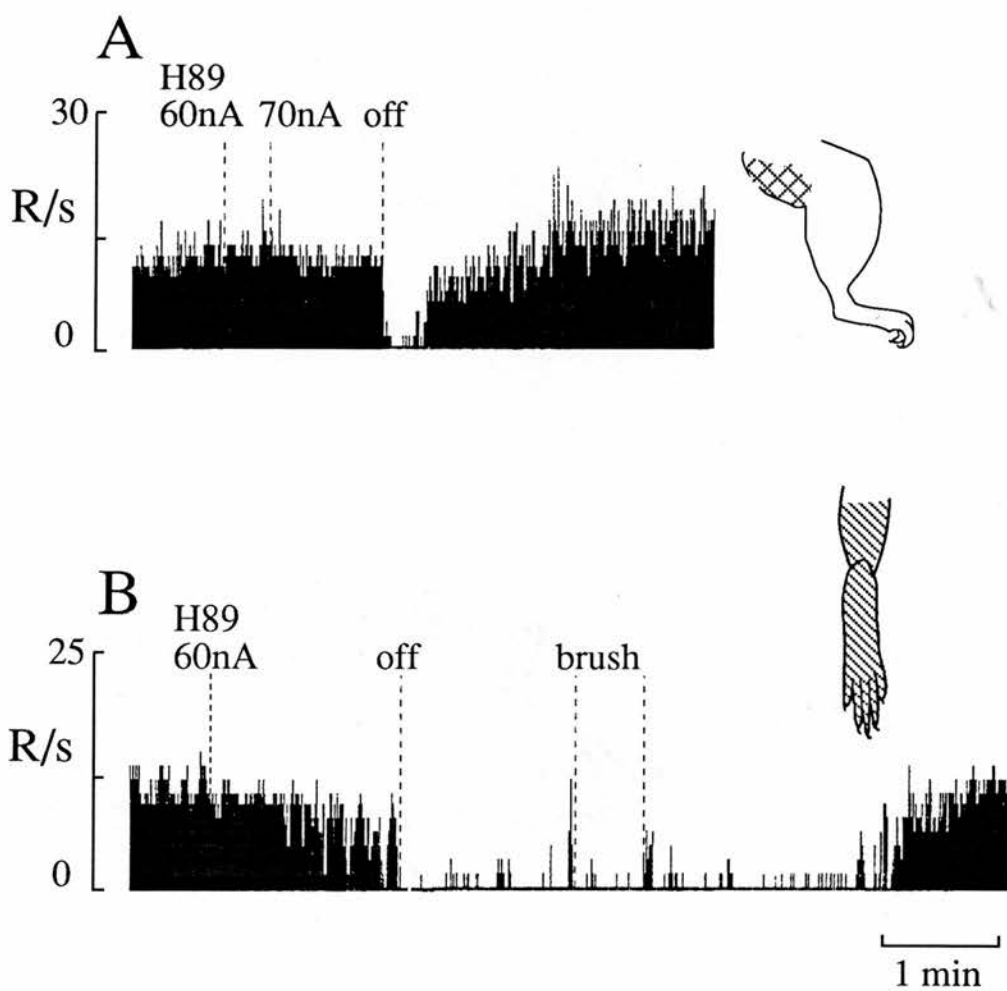


### **Figure 4.5b**

#### **The effect of ionophoretically applied H89, a protein kinase A inhibitor, on evoked activity in dorsal horn neurons**

Ionophoresis of another protein kinase A inhibitor, H89, resulted in similar changes. The records show ongoing firing of individual neurons, displayed as action potentials per second, integrated over 700 millisecond bins (R/s), plotted against time. The receptive field is shown on the inserts by diagonal lines, or hatched areas where it occurred on both sides of the leg.

Record (A) is an example of how ionophoresis of H89 led to a reduction in brush-evoked firing. This was typical in half of the neurons tested. Trace (B) demonstrates how, at a lower current, H89 also reduced the firing evoked by the ionophoretic application of metabotropic glutamate receptor agonist (1S,3R)-ACPD (-4nA). The broken lines in (B) represent 4 minutes which passed before recovery was observed.

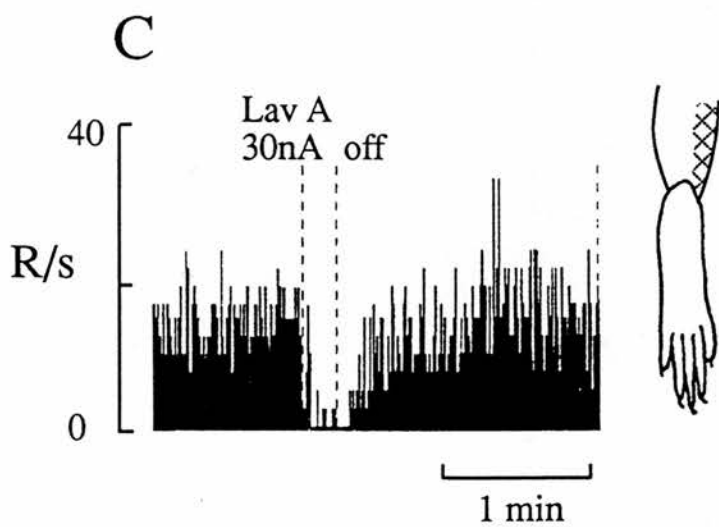
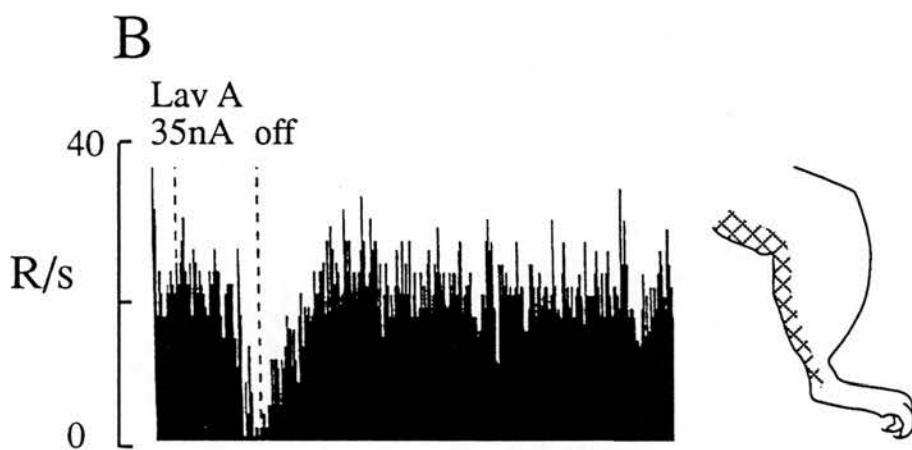
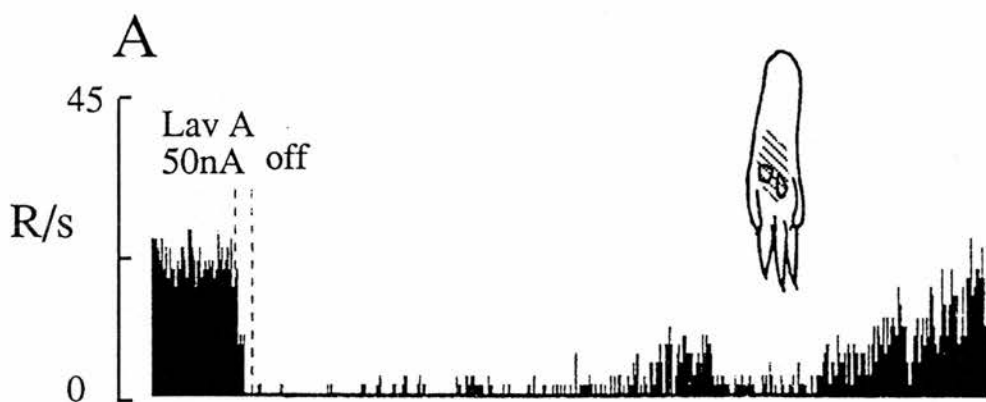


### **Figure 4.6a**

#### **The effect of ionophoretically applied Lavendustin A, a tyrosine kinase inhibitor, on evoked activity in dorsal horn neurons**

The records show ongoing firing of individual neurons, displayed as action potentials per second, integrated over 700 millisecond bins (R/s), plotted against time. Diagonal lines on the inserts show the receptive field of the cells, with hatched areas representing both sides of the leg/foot.

These are typical examples of how lavendustin A reduced firing evoked by (A) brush, (B) mustard oil and (C) metabotropic glutamate receptor agonist (1S,3R)-ACPD (-1nA).

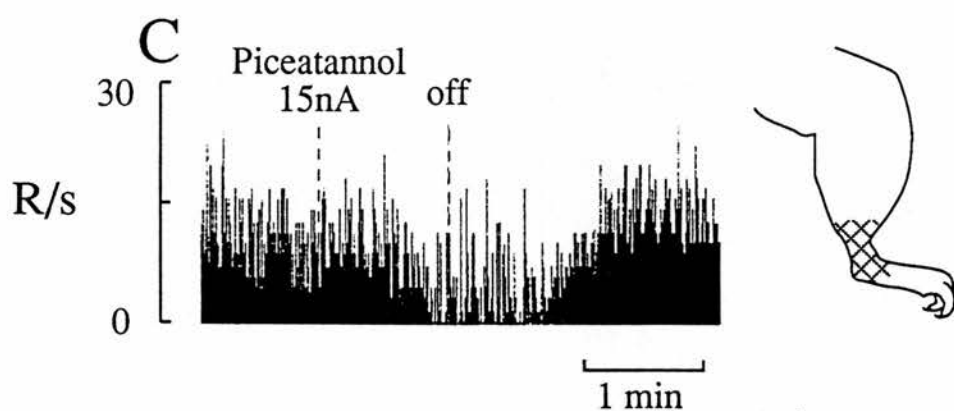
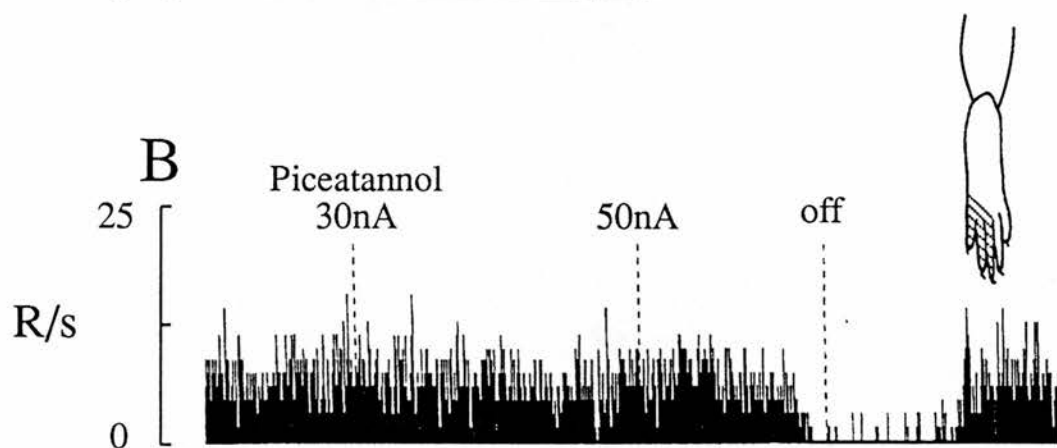
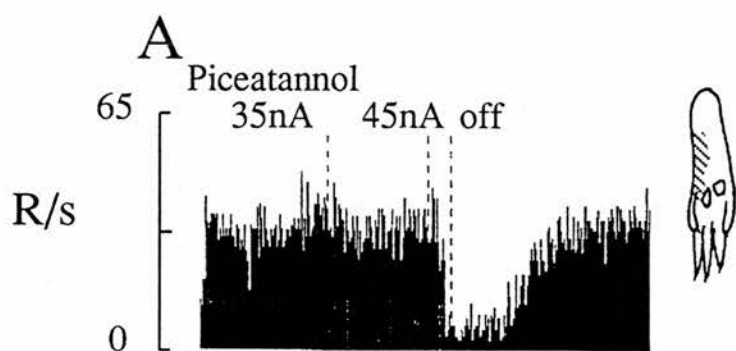


### **Figure 4.6b**

#### **The effect of ionophoretically applied Piceatannol, a tyrosine kinase inhibitor, on evoked activity in dorsal horn neurons**

A second tyrosine kinase inhibitor, piceatannol, resulted in similar alterations in evoked responses. The records show ongoing firing of individual neurons, displayed as action potentials per second, integrated over 700 millisecond bins (R/s), plotted against time. Diagonal lines on the inserts show the receptive field of the cells, with hatched areas representing both sides of the leg/foot.

These are typical examples of how piceatannol reduced firing evoked by (A) brush, (B) mustard oil and (C) metabotropic glutamate receptor agonist (1S,3R)-ACPD (-2nA).

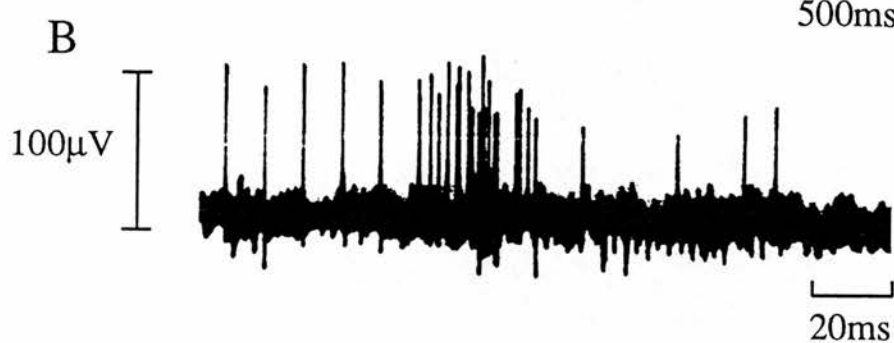
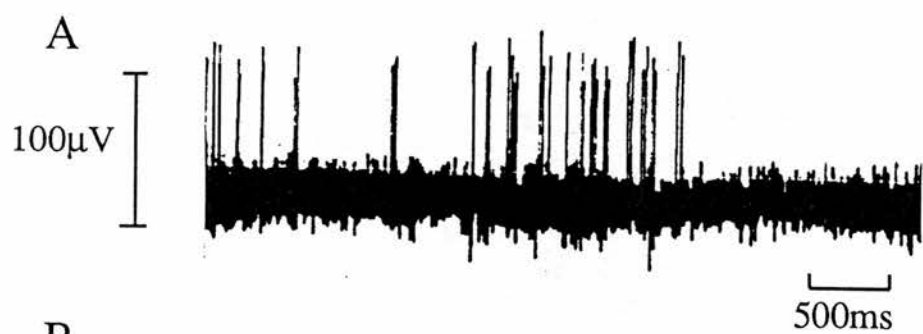


### **Figure 4.7**

#### **Oscilloscope records of the effect of intracellular signalling pathway inhibitors lavendustin A and KT5720 on individual dorsal horn neurons firing in response to the cutaneous application of mustard oil**

The records show a sudden cessation of mustard oil-evoked firing during the iontophoresis of (A) tyrosine kinase inhibitor lavendustin A (35nA) and (B) protein kinase A inhibitor KT5720 (20nA). In the upper trace a brief period of excitation can be seen preceding the inhibition of firing. The lower trace however, is an example of a reduction in spike height which often accompanied this phenomenon.





**Table 4.1**

**A summary of the effects of ionophoretically-applied inhibitors of intracellular signalling pathways on the activity of dorsal horn neurons evoked by brush, mustard oil or (1S,3R)-ACPD**

The ranges of ionophoretic currents required for the effects reported are indicated in parentheses below each drug. In the case of (1S,3R)-ACPD, higher currents ( $\leq 60\text{nA}$ ) were sometimes briefly used to initiate firing, but after the first few seconds, the elevated activity could always be maintained by  $\leq 10\text{nA}$ .

The effects of the drugs on evoked responses are indicated as the mean $\pm$ SEM percentage inhibition compared to pre-drug control activity. In each case, the number of neurons contributing to the data is shown below and in parentheses, the proportion (percentage) of those neurons which individually gave a clear response of  $>20\%$  inhibition. The statistical significance of drug-induced changes in firing rate was assessed using the Wilcoxon test on mean raw firing data in action potentials per second; comparing the 20 or 30 second period immediately prior to drug administration with the 20 or 30 second period centred on the greatest inhibitory effect of the drug ( $\ddagger$  represents  $p<0.05$ ; ns indicates not significantly different,  $p<0.05$ ).

The data from GF109203X and chelerythrine on brush- and mustard oil-evoked activity is taken from Munro *et al*, 1994.

		Inhibition of evoked activity (% inhibition compared to pre-drug controls)		
Principal Target	Drug	Brush	Mustard Oil	(1S,3R)-ACPD (0-10nA)
Calmodulin/ $\text{Ca}^{++}$ -dependent kinase II	Calmidazolium (30-60nA)	16±10 <i>n.s.</i> n=8 (25%)	42±14✂ n=7 (71%)	68±16✂ n=5 (80%)
	KN62 (30-70nA)	42± 13✂ n=12 (50%)	51± 19✂ n=5 (80%)	83± 9✂ n=5 (100%)
PKC	GF109203X (20-60nA)	3±3 <i>n.s.</i> n=5 (20%)	53±10✂ n=9 (89%)	86±12✂ n=5 (100%)
	Chelerythrine (10-80nA)		70±10✂ n=8 (100%)	84±11✂ n=7 (100%)
PLA <sub>2</sub>	ONO-RS-082 (30-65nA)	28±13 <i>n.s.</i> n=9 (44%)	70±11✂ n=5 (100%)	75±9✂ n=8 (100%)
	BS4 (30-60nA)	37±12✂ n=9 (56%)	74±11✂ n=5 (100%)	86±6✂ n=7 (100%)
PKA	KT5720 (20-60nA)	59±15✂ n=10 (70%)	73±13✂ n=7 (86%)	87±11✂ n=5(80%)
	H89 (30-70nA)	45±15✂ n=10 (50%)	66±10✂ n=5 (100%)	79±13✂ n=6 (100%)
Tyrosine Kinases	Lavendustin A (30-70nA)	60±12✂ n=11 (82%)	58±10✂ n=8 (88%)	69±12✂ n=7 (100%)
	Piceatannol (7-50nA)	48±15✂ n=13 (61%)	87±7✂ n=6 (83%)	84±9✂ n=6 (100%)

#### **4.3.3. EFFECTS OF INHIBITORS OF INTRACELLULAR SIGNALLING PATHWAYS ON NEURONAL RESPONSES TO NOXIOUS CHEMICAL STIMULATION BY MUSTARD OIL**

##### **4.3.3 (a) Calmodulin/ $\text{Ca}^{++}$ -dependent kinase II Inhibitors (Calmidazolium and KN62)**

Both of these compounds significantly reduced activity evoked by the cutaneous application of mustard oil (see Figs 4.2a and 4.2b). Calmidazolium (30-60nA) depressed neuronal activity in 5 out of 7 cells, by a mean inhibition on the population of  $42 \pm 14\%$  from control levels ( $p < 0.05$ , Wilcoxon test). Four of the 5 units recovered to between 75 and 100% of pre-drug levels, all within 5 minutes. The 2 cells which were not markedly inhibited showed very small increases in firing (+3 and +17% from control). KN62 (30-70nA) produced similar results, in that 4 of 5 tested units showed a marked decrease in firing, with a mean and significant reduction of  $51 \pm 19\%$  for the whole group ( $p < 0.05$ , Wilcoxon test). Of these 4 neurons, 1 failed to return to control firing rates (given upto 15 minutes). The remaining 3 cells did recover, within 2-5 mins. The final tested neuron was excited by 16%.

##### **4.3.3 (b) Protein Kinase C (PKC) Inhibitors (Chelerythrine and GF109203X)**

Munro *et al* (1994a) investigated the effect of GF109203X and chelerythrine on mustard oil-induced firing. They showed that both antagonists had a significant and reproducible effect on such neuronal activity. GF109203X (20-60nA) reduced firing in all but 1 of the 9 cells tested (mean inhibition of  $53 \pm 10\%$ ) and chelerythrine (10-80nA) reduced the response of all 8 units examined (a mean inhibition of  $70 \pm 10\%$ ;  $p < 0.05$ , Wilcoxon test).

##### **4.3.3 (c) Phospholipase $\text{A}_2$ (PLA $_2$ ) Inhibitors (ONO-RS-082 and BS4)**

A total of 10 cells were tested on mustard oil-evoked activity, 5 with each of the inhibitors. All neurons showed a profound reduction in firing when the antagonists were ionophoresed (see Figs 4.4a and 4.4b). ONO-RS-082 (30-65nA) significantly decreased activity by a mean of  $70 \pm 11\%$  ( $p < 0.05$ , Wilcoxon test) from control responses and BS4 (30-60nA) had very similar effects, in that it significantly reduced firing by  $74 \pm 11\%$  ( $p < 0.05$ , Wilcoxon test). Only 1 cell, in the ONO-RS-082 group, failed to recover from antagonist ionophoresis. Two of the units in the BS4 group only returned to within 50% of control firing levels.

#### **4.3.3 (d) Protein Kinase A (PKA) Inhibitors (KT5720 and H89)**

Twelve neurons were investigated. In 6 out of 7 cells KT5720 (20-60nA) profoundly inhibited mustard oil-evoked activity by  $73\pm13\%$  ( $p<0.05$ , Wilcoxon test). Three of these 6 units recovered (see Fig 4.5a), whereas 3 showed no activity during the next 10-15 minutes of the experiment. Activity in the seventh cell was not changed from pre-drug levels by the ionophoresis of KT5720. H89 (30-70nA) brought about a considerable reduction in neuronal firing from control values in all 5 which were tested (see Fig 4.5b), by a mean of  $66\pm10\%$  ( $p<0.05$ , Wilcoxon test). Recovery (75-100%) was observed in all examples.

#### **4.3.3 (e) Tyrosine Kinase Inhibitors (Lavendustin A and Piceatannol)**

Similar to the effect on brush-evoked firing, lavendustin A (30-70nA) significantly reduced mustard oil-evoked activity from control levels across the population of cells (markedly in 7 of 8 units; see Fig 4.6a), by a mean of  $58\pm10\%$  ( $p<0.05$ , Wilcoxon test). The cell which was not notably inhibited showed a 7% decrease in firing. Four of the 7 inhibited cells showed recovery close to pre-drug levels, whereas the remaining 3 did not show a rise in firing within 10-15 minutes. Piceatannol (7-50nA) produced comparable effects by clearly inhibiting 5 out of 6 cells, all of which recovered to within 75% of pre-drug activity (see Fig 4.6b), with a mean decrease of  $87\pm7\%$  ( $p<0.05$ , Wilcoxon test). The sixth cell showed no discernable change in activity during application of the compound.

### **4.3.4. EFFECTS OF INHIBITORS OF INTRACELLULAR SIGNALLING PATHWAYS ON NEURONAL RESPONSES TO IONOPHORESIS OF MGLU RECEPTOR AGONIST (1S,3R)-ACPD**

#### **4.3.4 (a) Calmodulin/ $\text{Ca}^{++}$ -dependent kinase Inhibitors (Calmidazolium and KN62)**

All 10 cells in this group responded to innocuous brushing and noxious pinching of the receptive field. In 4 of 5 cells calmidazolium (30-60nA) markedly reduced the firing evoked by ionophoresis of (1S,3R)-ACPD (see Fig 4.2a), by a population mean of  $68\pm16\%$  ( $p<0.05$ , Wilcoxon test). In the fifth cell there was a slight elevation in firing (+10% from pre-drug activity). In all of a further 5 units KN62 significantly inhibited the response by a mean of  $83\pm9\%$  ( $p<0.05$ , Wilcoxon test). See Fig 4.2b.

#### **4.3.4 (b) Protein Kinase C (PKC) Inhibitors (Chelerythrine and GF109203X)**

Of the 12 units tested, 4 responded only to brushing of the receptive field. As well as responding to brush, 5 showed increased activity to noxious pinching and 1 to noxious heating. Three did not have their sensory inputs characterised. Ionophoresis of chelerythrine (10-80nA, n=7) and GF109203X (20-60nA, n=5) significantly reduced (1S,3R)-ACPD-evoked activity in all cases (see Fig 4.3), with mean inhibitions of  $84\pm11\%$  and  $86\pm12\%$  respectively (both  $p<0.05$ , Wilcoxon test).

#### **4.3.4 (c) Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) Inhibitors (ONO-RS-082 and BS4)**

Two of these 15 units responded only to brushing of the receptive field, 8 were not characterised further and the remaining 5 responded to noxious pinch. In 8 out of 8 cases ONO-RS-082 (30-65nA) profoundly decreased (1S,3R)-ACPD-evoked firing levels (see Fig 4.4a), with a mean reduction of  $75\pm9\%$ . Similarly, BS4 (30-60nA) caused all 7 tested units to be inhibited (see Fig 4.4b), the mean fall being  $86\pm6\%$  (both  $p<0.05$ , Wilcoxon test).

#### **4.3.4 (d) Protein Kinase A (PKA) Inhibitors (KT5720 and H89)**

Eight of the 11 units in this group responded to noxious pinch as well as innocuous brushing of the receptive field. The other 3 cells were not characterised beyond their response to brush. Ejection of KT5720 (20-60nA) and H89 (30-70nA) resulted in a marked inhibitory effect on (1S,3R)-ACPD-evoked activity in all but 1 cell. KT5720 (n=5) decreased firing across the population by a mean of  $87\pm11\%$  ( $p<0.05$ , Wilcoxon test). In one neuron this compound had a dual effect in that it caused a facilitation ( $>200\%$ ) in firing at 20nA and a reduction of 85% at 40nA. H89 clearly reduced the firing of all 6 tested cells, by a mean of  $79\pm13\%$  ( $p<0.05$ , Wilcoxon test). See Figs 4.5a and 4.5b.

#### **4.3.4 (e) Tyrosine Kinase Inhibitors (Lavendustin A and Piceatannol)**

Four of the cells tested with (1S,3R)-ACPD responded positively to noxious pinching of the skin, 1 to noxious heating and the remainder of the group were not tested beyond their response to brushing of the receptive field. Of the 7 units in which lavendustin A (30-70nA) was tested, activity was decreased by a mean of  $69\pm12\%$  from pre-drug controls (see Fig 4.6a). Likewise, piceatannol (7-50nA)

inhibited 6 out of 6 tested neurons (see Fig 4.6b), with a mean reduction of  $84\pm9\%$  (both  $p<0.05$ , Wilcoxon test).

As with the results in Chapter 3, side-by-side comparisons of the effect of each compound on all three types of evoked activity could not be carried out as a consequence of the lasting effects induced by mustard oil. However, several comparisons were made on the population of neurons tested. In the case of CamKII, PKC and PLA<sub>2</sub> inhibitors, but not the other agents, there appeared to be a lesser effect on brush-evoked activity than on the other responses. The mean percentage inhibition of mustard oil- and (1S,3R)-ACPD-evoked responses by CamKII inhibitor calmidazolium, and the PLA<sub>2</sub> inhibitors ONO-RS-082 and BS4 were consistently two-fold greater than their effect on brush-evoked responses, and in the case of the PKC inhibitor GF109203X, it was many fold more. Furthermore, comparisons between two types of evoked activity were possible in some cases. For example, in the case of a single neuron tested with BS4, direct comparison on brush and (1S,3R)-ACPD responses revealed a selective inhibition of the latter (see Figure 4.4b), in accordance with the overall population of neurons investigated. Similarly, piceatannol caused inhibition of responses to both brush and mustard oil when directly compared on a single cell, again typical of the overall population.

A summary of these results can be found in Table 4.1, where data are given as the mean $\pm$ SEM percentage inhibition in the total population of cells tested. The results are presented in a manner which shows how each sensory input was affected by the range of inhibitors. However, the effect of individual compounds on all three inputs can be compared in figures 4.2 - 4.6.

One phenomenon which occurred occasionally during evoked activity was a very abrupt end in the firing of the neuron. This was sometimes preceded by a slight excitation and/or reduction in spike height, showing characteristics similar to a depolarisation block (see Fig 4.7). Recovery was not seen in these instances. This occurred throughout the range of compounds tested, although KT5720 showed more cases than any other drug, and was not specific to the type of activity evoked. The following shows which drugs induced this type of response and, in parentheses, the frequency with which it occurred:

Brush:                      KN62 (1/13); BS4 (1/10); KT5720 (3/13); H89 (1/11);  
                                    lavendustin A (2/13); piceatannol (3/16)



Mustard oil:	calmidazolium (1/8); KT5720 (3/10)
(1S,3R)-ACPD:	calmidazolium (1/6); KN62 (1/6); chlerythrine (1/8); KT5720 (2/7); piceatannol (1/7)

#### 4.4. DISCUSSION

Extracellular recordings were made from neurons in laminae III-V of the dorsal horn, data based on depth readings from the microdrive attached to the electrode, along with subsequent blue spot histology carried out on the spinal cord. The majority of units in this study were multireceptive, with a number of non-nociceptive neurons in the brush and agonist experiments. The type of cell did not appear to have any effect on the outcome of pharmacological tests. All cells displayed very low basal firing rates of less than 1Hz prior to activity evoked by brush, mustard oil or (1S,3R)-ACPD. Frequent vehicle and current controls revealed no reproducible effects of the ionophoresis of either 1M NaCl or 0.5% DMF on the evoked activities discussed. Following the characterisation of the role of mGluRs in sensory processing, utilising innocuous brush- and noxious mustard oil-evoked activity in dorsal horn neurons (see Chapter 3) the same model was subsequently used to investigate the mechanism(s) by which they may act.

Of the five signal transduction targets which were investigated, protein kinases A and C (PKA; PKC), phospholipase A<sub>2</sub> (PLA<sub>2</sub>), non-receptor tyrosine kinases and the Ca<sup>++</sup>/calmodulin-dependent kinase II (CamKII), all showed an apparent link with whichever of the group I/II mGluRs involved here, as their inhibitors caused distinct reductions in the activity evoked by the ionophoresis of mGluR agonist (1S,3R)-ACPD. These decreases appear not to be non-selective effects for several reasons: not all evoked activity could be reduced by the ionophoresis of these second messenger inhibitors (eg brush-evoked activity in a number of instances, notably in the cases of PKC, PLA<sub>2</sub> and CamKII)) Furthermore, (1S,3R)-ACPD-elicited neuronal activity cannot be reduced by the co-ionophoresis of any drug, as Chapter 3 demonstrated how D-AP3 could not alter firing evoked by this agonist. However, as some of the compounds used were equally effective in reducing all evoked responses the possibility of a non-selective action, or one secondary to the inhibition of the specific signal transduction targets, cannot be excluded. Therefore the link between mGluRs and PKA or non-receptor tyrosine kinases in particular is not conclusive. The present study still however provides evidence that mGluR activation is likely to involve the mobilisation of several



second messengers and their subsequent pathways. This evidence is consistent with previous experiments which have shown the ability of mGluR agonists to induce various signal transduction pathways (see section 1.3.3.(b)).

Some of the signal transduction antagonists tested in this study demonstrated a preferential inhibition of mustard oil-, rather than brush-evoked responses of dorsal horn neurons. Calmidazolium, an inhibitor of the  $\text{Ca}^{++}$ -binding protein calmodulin (thence  $\text{Ca}^{++}$ /calmodulin-dependent kinase II; CamKII), ONO-RS-082, a  $\text{PLA}_2$  inhibitor and the PKC antagonist GF109203X had no significant effect on the brush-evoked responses observed in the population of neurons tested. Also, the inhibitory effect of an additional  $\text{PLA}_2$  inhibitor, BS4, was consistently two-fold greater on agonist and mustard oil responses than on activity induced by innocuous brushing of the hindlimb. Along with the data from the agonist experiments, these results are in agreement with the hypothesis of a phosphoinositide-hydrolysing-linked Group I mGluR participating in the mediation of sustained C-fibre responses evoked by the application of mustard oil.

The consequences of mGluR activation are likely to be widespread, as these targets which demonstrate a modality-selective role represent stages in merely three of many possible different pathways which can be activated by a single initial step which generates  $\text{IP}_3$  (see Figure 1.3.(a)). Activation of Group I mGluRs can result in phosphoinositide hydrolysis (Nicolletti *et al*, 1986; Challis *et al*, 1988; Sladeczek *et al* 1988; Baird *et al*, 1991) to produce  $\text{IP}_3$ , with subsequent rises in intracellular calcium (Murphy & Miller, 1988; 1989). On the other arm of the phospholipase C (PLC) cascade (see Figure 1.3.(a)) there will be mGluR-linked activation of diacylglycerol with the subsequent mobilisation of PKC (Manzoni *et al*, 1990; Behnisch & Reymann, 1993), thereby potentially leading to  $\text{PLA}_2$  activation and a release of arachidonic acid (Aramori & Nakanishi, 1992). Downstream modulation of various ion channel activity by mGluR agonists has also been reported (Lester & Jahr, 1990; Fagni *et al*, 1991), including in spinal cord neurons (Bleakman *et al*, 1992; Cerne & Randic, 1992). Longer-term alterations, for example due to immediate-early gene activation, may be initiated as a result of these pathways being implemented. The data from the present experiments highlights the roles of PKC,  $\text{PLA}_2$  and CamKII in the mediation of nociceptive inputs, via one or more mGluRs which can be activated by (1S,3R)-ACPD. Since all three of these second messengers are generally downstream of  $\text{IP}_3$  or diacylglycerol production, it is likely that the mGluR involved is of Group I in origin, i.e. mGluR1 and/or 5, which are coupled to the activation of PLC.

The activity evoked by the ionophoresis of mGluR agonist (1S,3R)-ACPD was markedly reduced, without exception, by the co-ionophoretic application of both PKC inhibitors, GF109203X and chelerythrine. This decrease in activity matched that observed on mustard oil-, but not innocuous brush-evoked responses by Munro *et al* (1994a). This data is highly indicative of an mGluR-associated role for PKC in the selective transmission of C-afferent fibre inputs. The prominent blockade of mustard oil-evoked activity by PKC inhibitors is congruent with the translocation/activation of dorsal horn PKC observed by measuring PDBu-binding, following mustard oil- and formalin-induced inflammation, as well as after a chronic constrictive nerve injury (CCI) (Mao *et al*, 1992; 1993; Munro *et al*, 1994a; Tölle *et al*, 1994; Yashpal *et al*, 1995). In further support of a role for PKC in mediating nociception, behavioural testing has demonstrated how persistent nociceptive responses elicited by the subcutaneous administration of formalin can be attenuated by PKC inhibitors (Coderre, 1992; Coderre & Yashpal, 1994; Yashpal *et al*, 1995), as can the prolonged nociceptive behaviour induced by CCI (Hayes *et al*, 1992). Interestingly, Yashpal *et al* (1995) observed a matched GF109203X- and chelerythrine-induced reduction in the contralateral mechanical hyperalgesia resulting with the formalin-evoked ipsilateral hyperalgesia. In addition to the evidence from nociception and pain studies, the sensitivity of LTP in hippocampal cells to inhibition of PKC (Lovinger *et al*, 1987; Malinow *et al*, 1988; 1989; Malenka *et al*, 1989; Bliss & Collingridge, 1993) and the requirement of PKC activation in memory processing (Olds *et al*, 1989) are further common features between this phenomenon and the analogous sensitisation which occurs in dorsal horn neurons following a sustained noxious input. Therefore the data from previous experiments which implicate PKC in the mediation of nociceptive, but not non-nociceptive inputs (Munro *et al*, 1994a) can now be integrated with the results from the present study to suggest that this mediation can occur, at least in part, via a Group I mGluR. There is of course the possibility of other receptors having a link with PKC, among them being the neurokinin receptors. Urban *et al* (1994) found that an NK<sub>2</sub>, though not NK<sub>1</sub> agonist-induced VRPs could be blocked by staurosporine, a PKC and PKA inhibitor, thus indicating the possibility of PKC involvement in NK<sub>2</sub> receptor-mediated depolarisation. In addition to this, the intravenous administration of non-peptide NK<sub>2</sub> receptor antagonist SR48968 prevented the *in vivo* translocation of PKC normally observed following the cutaneous application of mustard oil (F.E. Munro PhD thesis, 1995). As mentioned previously, ion channel receptors can be modulated downstream of second messengers. PKC is a good example of this phenomenon, as phorbol ester activators of this kinase can facilitate responses elicited by NMDA in

dorsal horn neurons *in vitro* (Gerber *et al*, 1989). Rusin *et al* (1992) observed a reversal of SP-induced facilitation of NMDA responses when staurosporine was administered, this may have been due to PKC, or perhaps PKA inactivation. Thus there is the possibility of a cellular interaction at the second messenger level, as a consequence of the simultaneous activation of EAA and other receptors.

As well as a selective role for PKC in nociception being established (Munro *et al*, 1994a), the current investigation has provided substantial evidence for a specific role of PLA<sub>2</sub>. The activity evoked in dorsal horn neurons by either the cutaneous administration of mustard oil, or by ionophoretic application of (1S,3R)-ACPD could be markedly reduced when PLA<sub>2</sub> inhibitors ONO-RS-082 and BS4 were ionophoresed. However, in the case of the responses elicited by light innocuous brushing of the hindlimb, the effect on the population of neurons tested with ONO-RS-082 was not significant. Furthermore, the inhibitory effect on brush responses by BS4, which was significant in the overall population was only considerable in just over half of the neurons on which it was tested. The data here would therefore suggest that PLA<sub>2</sub>, like PKC, plays a distinct role in the mediation of sensory inputs, and that this can be, at least in this instance via mGluRs. This enzyme can be activated downstream of PKC and is responsible for the generation of arachidonic acid (AA) and its subsequent metabolites, including the prostaglandins (PGs). Until recent years the role of these agents was thought to lie primarily in the periphery, with some of the most common analgesics and anti-pyretics used today targeting this pathway (non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenase (COX), the enzyme responsible for the conversion of AA to PGs). However some recent experiments have highlighted a capacity for these signal transduction mechanisms to operate in central structures. When administered intrathecally, NSAIDs can diminish the activity evoked in dorsal horn neurons by the subcutaneous injection of formalin (Chapman & Dickenson, 1992). In accordance with this, the behaviour which results from such formalin administration can be markedly increased or decreased in animals which receive intrathecal AA or NSAIDs respectively (Malmberg & Yaksh, 1992b; Yashpal &Coderre, 1993; Coderre & Yashpal, 1994). Additionally, baseline paw withdrawal latencies were not affected by an inhibitor of AA (Coderre & Yashpal, 1994), thus indicating a specific role for this signal transducer in a sensitised state. The behavioural hyperalgesia which is elicited when NMDA, AMPA and SP are intrathecally administered can also be decreased when NSAIDs are applied (Malmberg & Yaksh, 1992a). Similarly formalin-evoked responses which have been facilitated by SP or glutamate are reduced by an inhibitor of AA (Coderre & Yashpal, 1994). It therefore seems

apparent that nociceptive inputs to the spinal cord are at least partially mediated by PLA<sub>2</sub> and the pathways in which it is involved. The current study re-iterates this fact and moreover provides evidence that this can be brought about by an mGluR, which is activated by (1S,3R)-ACPD. With the use of newly selective antagonists Boyce *et al* (1994) demonstrated that carrageenan-induced hyperalgesia can be reduced when the action of COX2 is inhibited, without producing gastric ulceration, a common side-effect of general COX inactivation. This may provide an even more discrete downstream target in this cascade at which to target novel analgesics.

One of the most selective compounds observed in this investigation was the CamKII inhibitor calmidazolium. This drug prevents calmodulin from binding to Ca<sup>++</sup>, and thus the activation of the kinase in question, an event which would normally result in the further phosphorylation of many other proteins, including autophosphorylation of CamKII subunits in the rat brain (McGuinness *et al*, 1985; Miller & Kennedy 1985; 1986). Inhibition of this step appears to have specifically interrupted the transmission of sustained C-, but not A $\beta$ -afferent fibre inputs to dorsal horn neurons. A switch-like role for this CamKII has been suggested, and its kinetics have led to the hypothesis that it has the ability to encode long-term alterations in cellular activity (Lisman & Goldring, 1988). The present data are consistent with this hypothetical role. Furthermore the results here give an insight into the likely nature of this sustained input, i.e. it would appear that activity of a high intensity is necessary. A role for CamKII in sustained high threshold synaptic inputs is substantiated by evidence from LTP studies. Inhibition of this kinase results in the inability of hippocampal cells to induce LTP, though once it is established CamKII inhibitors are no longer effective (Malenka *et al*, 1989; Malinow *et al*, 1989). In addition to this, Silva *et al* (1992) examined genetically-engineered mice which did not express CamKII, to reveal that LTP could not be evoked in the hippocampus. Behavioural testing showed these mice were otherwise normal, thus indicating a specific role in this prolonged synaptic sensitisation phenomenon. Unfortunately no nociceptive testing was carried out, as it would be very interesting to know the effect of this knock-out on pain thresholds, and the ability of known analgesics to affect the responses.

In the present study the PKA inhibitors KT5720 and H89 were equally effective in inhibiting all types of evoked activity in dorsal horn neurons. This could imply that either the compounds were acting in a non-selective manner, or that PKA phosphorylation is indeed involved in the transmission of both innocuous and noxious information. Group II and III mGluRs are negatively linked to cAMP formation (Manzoni, *et al*, 1990; Cartmell *et al*, 1992; Schoepp & Johnson, 1992; see



Figure 1.3.(b)). No evidence from the present investigation supports the hypothesis that one of these cAMP inhibiting mGluRs is found in the spinal cord, otherwise the ionophoresis of PKA inhibitors might have been expected to mimic, rather than inhibit the agonist-evoked responses. However, the inhibition of (1S,3R)-ACPD-induced activity by both KT5720 and H89 is actually consistent with the hypothesis that there are also (1S,3R)-ACPD-activated mGluRs which are positively linked to cAMP production (Aramori & Nakanishi, 1992; Winder & Conn, 1992; 1993). In further support of a possible role of PKA here, this enzyme has been implicated in prolonged synaptic events such as LTP (Chetkovitch *et al*, 1991). Furthermore, the adenylate cyclase activator forskolin can facilitate the neuronal responsiveness of dorsal horn neurons to NMDA (Cerne *et al*, 1993). Although this cumulative evidence may suggest the involvement of this kinase in sustained synaptic inputs, the possibility of non-selective actions of these drugs makes it difficult to derive any further conclusions as to a role for PKA in the transmission of sensory information at the level of the spinal cord. The data obtained using these compounds may in fact be representative of effects secondary to the inhibition of PKA, although both KT5720 and H89 are highly selective inhibitors of PKA activity (Kase *et al*, 1987; Chijiwa *et al*, 1990). This may also be the case for the non-receptor tyrosine kinase inhibitors which were investigated. Activity evoked by all three inputs, brush, mustard oil and agonist, could be equally reduced by the application of piceatannol and lavendustin A. These compounds have demonstrated high selectivity for non-receptor tyrosine kinases (Geahlen *et al*, 1989; Hsu *et al*, 1991) and it is therefore possible that the interruption of all evoked activity was due to a genuine inhibition of these kinases, particularly as they both behaved in a similar manner. A previous intracellular recording study investigating LTP in the hippocampus, demonstrated how bath-applied and post synaptically-injected lavendustin A (10 $\mu$ M), and a further tyrosine kinase inhibitor, genistein, could block LTP initially, though not once it was established and neither compound affected normal synaptic transmission (O'Dell *et al*, 1991). From this LTP study it may be expected that mustard oil-evoked activity could likewise remain unaffected once established. Since this was not the case, it must be assumed the role of tyrosine kinases in prolonged synaptic events in the hippocampus and spinal cord are somewhat different (though the two events have many other similarities) or that the drug effects here are non-specific. This data nonetheless indicates, at least in the case of lavendustin A, that tyrosine kinase inhibitors are capable of selective inhibition of some cellular events. Furthermore, since both compounds in the present study yielded similar results (and they are

structurally quite different) the data should not necessarily be discounted on the basis of the likelihood of non-specific effects.

Interestingly, the three pathways investigated in this study which appear to be specifically linked to mustard oil-evoked activity in dorsal horn neurons are all downstream of the activation of Group I mGluRs. This substantiates further the hypothesis in Chapter 3, that it is mGluR1 and/or 5 which selectively mediate noxious inputs. It would be most interesting to now observe the effect of intrathecally applied second messenger inhibitors on behavioural responses. In the case of those yielding general inhibitory effects in the present study, there may be the possibility of testing them on other reflexes, in order to re-assess their role in sensory inputs. Also, different models of sustained nociception, such as carrageenan- or CFA-induced inflammation may provide further information as to the specific roles of these inhibitors. In light of the numerous studies on the role of second messengers in LTP, and since many inhibitors affect the induction but not maintenance of this phenomenon, it may be very advantageous to assess the role of the signal transducers in the induction of the sensitisation process which occurs in dorsal horn neurons following a sustained noxious input.

In summary, the present data suggest that PKC, PLA<sub>2</sub> and CamKII are highly likely to play a role in the mediation of mustard oil-evoked activity in dorsal horn neurons. However, due to the inability to rule out non-specific events, the same cannot be concluded about non-receptor tyrosine kinases and PKA. These two signalling pathways may however play a genuine role in mediating a broad range of sensory inputs to the spinal cord. Furthermore, in this model of sustained nociception the signal transduction events discussed are likely to be linked at least in part to the activation of an mGluR (with the results from Chapter 3 indicating this to be mGluR1 and/or mGluR5).

## **CHAPTER 5:**

### **THE EFFECT OF INTRATHECALLY APPLIED METABOTROPIC GLUTAMATE AND TACHYKININ RECEPTOR ANTAGONISTS ON BEHAVIOURAL NOCICEPTIVE REFLEXES**

#### **5.1. AIMS**

Electrophysiological experiments, including the present study, have demonstrated a role for metabotropic glutamate (mGlu) and neurokinin (NK) receptors in various nociceptive inputs. In order to evaluate the possible corresponding role of mGlu, NK<sub>1</sub> and NK<sub>2</sub> receptors as analgesics in the behaving animal, the effect of intrathecally applied antagonists on behavioural responses to noxious mechanical and noxious thermal stimuli was assessed. These effects were investigated in the normal paw, and in separate groups of animals with a paw which had been inflamed by an intraplantar injection of carrageenan. Each antagonist was tested individually, as well as part of a co-injection regime in order to determine any interaction between mGlu and NK receptors.

#### **5.2. METHODS**

##### **5.2.1. Animals**

Animals were Glaxo-bred male and female Lister-hooded rats (30 - 70g). They were housed in steel bar based cages in a similar manner to that described in 3.2.

##### **5.2.2. Drugs**

All drugs were administered as a 10µl injection. L- and D-AP3 (30 and 60nmol), (S)-CHPG (24 and 48nmol) and GR82334 (0.1, 0.3 and 3nmol) were all made up in sterile saline. L659,874 (0.1, 0.3 and 3nmol) was dissolved in 0.3% dimethylsulphoxide (DMSO) in saline. The pH of each solution was adjusted to 7.2 - 7.4. All drug solutions were made by diluting frozen aliquots of stock, either in sterile saline or DMSO, immediately prior to the behavioural testing. Once thawed, they were not refrozen.

### 5.2.3. Intrathecal injections

Animals received a single 10µl intrathecal (i.t.) injection by means of a 10µl Hamilton microsyringe fitted with a disposable 30G needle. The correct positioning of the needle, around L5 - L6, was determined by a tail flick response. Immediately after the injection the animals were placed in a perspex box with a layer of sawdust on its base. They were allowed to move freely between tests.

### 5.2.4. Behavioural Assessment

Baseline measurements of paw withdrawal threshold/latency to noxious mechanical/thermal stimulation were taken at least 15 minutes prior to drug administration. Two thresholds/latencies for the right hindlimb were measured, if these differed by more than 1.0 units/seconds then a third was also taken, and the mean value used as baseline.

Following the intrathecal injection of drug or vehicle paw withdrawal reactions were tested at 1, 3 and 5 minutes. At the end of the test the animal was humanely destroyed.

#### 5.2.4.(a) *Behavioural Reflex to Noxious Mechanical Stimulation*

Paw withdrawal threshold to a noxious mechanical stimulus was measured using an Analgesy-Meter from Ugo Basile, 21025 Comerio - Varese, Italy. The animal's paw was placed under a plastic cone which provided increasing pressure, controlled by means of a foot switch, until the paw was withdrawn. The rise in pressure was brought about by a 10g weight moving along a rotating bar, which was located above a numerical scale (see figure 5.1.). The reading taken from the Analgesy-Meter (called "units" in the results section) was representative of grams x 10, i.e. 8 = 80g, the maximum being 250g. Since the animals used were very small, the withdrawal was often observed as a pulling action, and was gauged by the experience of the handler.

#### 5.2.4.(b) *Behavioural Reflex to Noxious Thermal Stimulation*

The apparatus used for the measurement of paw withdrawal latency to noxious heat was a Ugo Basile Tail Flick Unit. The dorsal, hairy surface of the paw was loosely held over a 50W infra-red bulb (see figure 5.2.) with a foot switch to turn it on, simultaneously activating a digital seconds display. When the rat retracted its paw the timer, and thus the heat source, automatically stopped due to an electronic circuit being interrupted because of lack of energy reflected by the foot. Latencies were recorded to the nearest 0.1 second, with a cut-off of 23.0 seconds.



### **5.2.5. Induction of Inflammation**

Separate groups of animals were used to assess the effects of drugs in the inflamed state, i.e. not the contralateral paw in the normal groups. In these animals, inflammation was induced in one hindpaw by the administration of lambda carrageenan. Prior to the induction of this inflammation, baseline responses were recorded for each animal. A 2% lambda carrageenan (Sigma Chemical Company, Poole, UK) solution was made up the previous day in sterile saline and left overnight at 4°C. A 100µl unilateral injection of this carrageenan was administered subcutaneously into the dorsal surface of the right (ipsilateral) hindpaw, using a disposable 21G needle and 1ml syringe, under a brief (one minute) halothane anaesthesia (one third O<sub>2</sub>, two thirds N<sub>2</sub>O, 2% halothane). Previous studies (Iadorola *et al*, 1988a; 1988b) have demonstrated how thermal hyperalgesia and oedema are at their maximum 4 hours after the carrageenan injection, even though they can persist for up to 4 days. The animals were therefore returned to their cages, where they could move around freely, and left for 3 (to 4) hours before withdrawal latencies were remeasured. Any reduction in paw withdrawal latencies at this time was taken as an indication of hyperalgesia. Paws were seen to be swollen and local oedema was apparent.

### **5.2.6. Controls**

Rats which received a 10µl injection of either sterile saline or 0.3% DMSO were tested in both the non-inflamed and the inflamed state.

### **5.2.7. Analysis of Data**

All paw withdrawal latencies were calculated as a percentage of those determined before the administration of vehicle or drug (represented by 'time 0' on graphs). The data shown are mean±SEM values for the group of subjects, however each rat had its own percentage change calculated, for all time points.

In separate groups of animals in which inflammation was induced, 'time -1' represents basal paw withdrawal latencies preceding the injection of lambda carrageenan, and is used to determine a shift indicative of hyperalgesia, 3 hours after such an injection. Readings from 'time 0' in this group are therefore 3 hours after the induction of inflammation and immediately prior to intrathecal injections.

Drug effects were compared to vehicle controls and statistical significance was determined using the Mann-Whitney U-test.  $P < 0.05$  was considered significant.

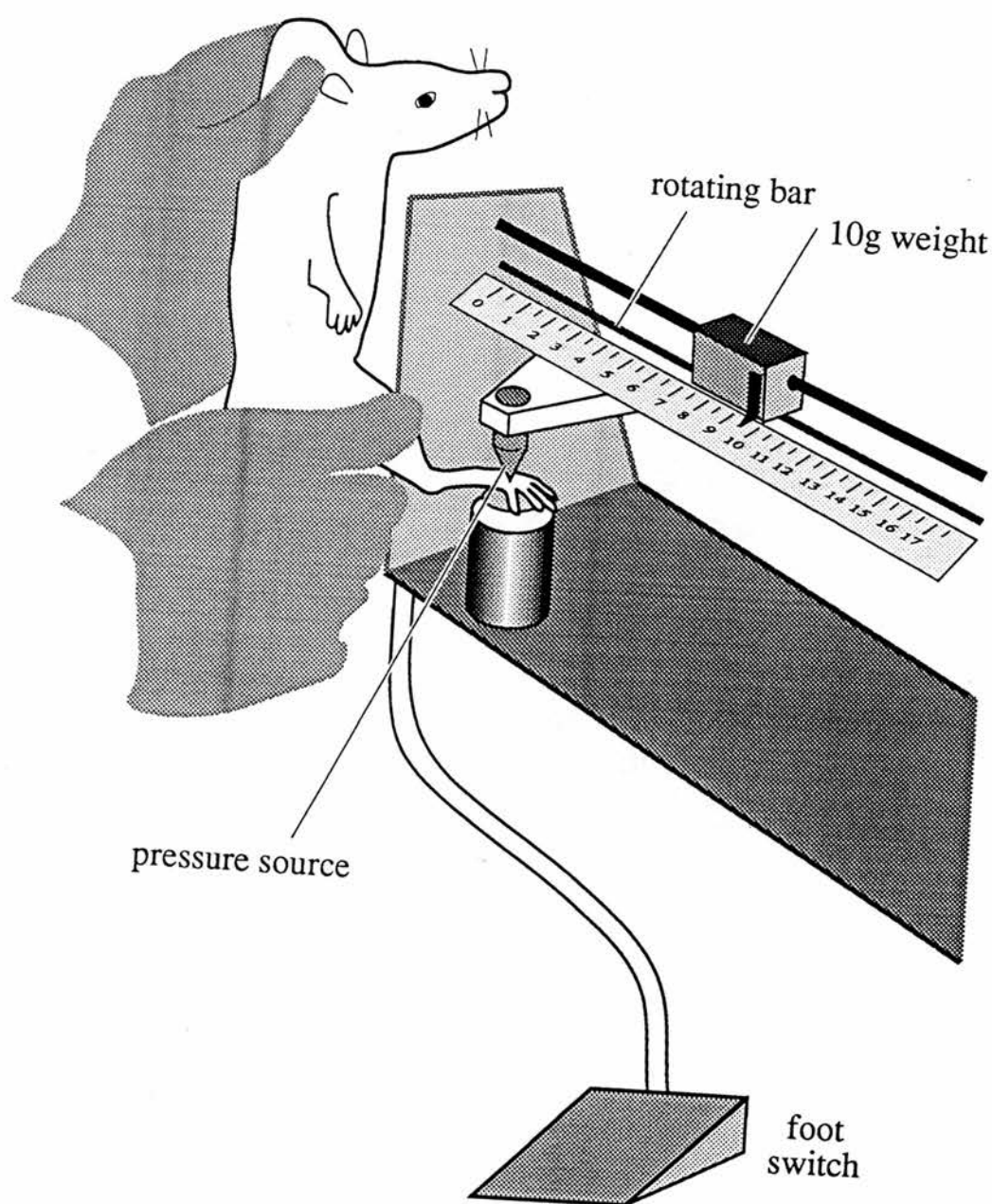
### **Figure 5.1**

#### **Schematic representation of the procedure for testing paw withdrawal threshold to noxious mechanical stimulation**

The animal had its paw placed under a plastic cone which provided an increasing pressure controlled by a foot switch.

A 10g weight rested in the groove of a rotating rod. When the foot switch was activated the motor-controlled rotating bar moved the 10g weight (attached to a second bar) along the groove by means of a screw-gear, with the pressure increasing at a constant rate.

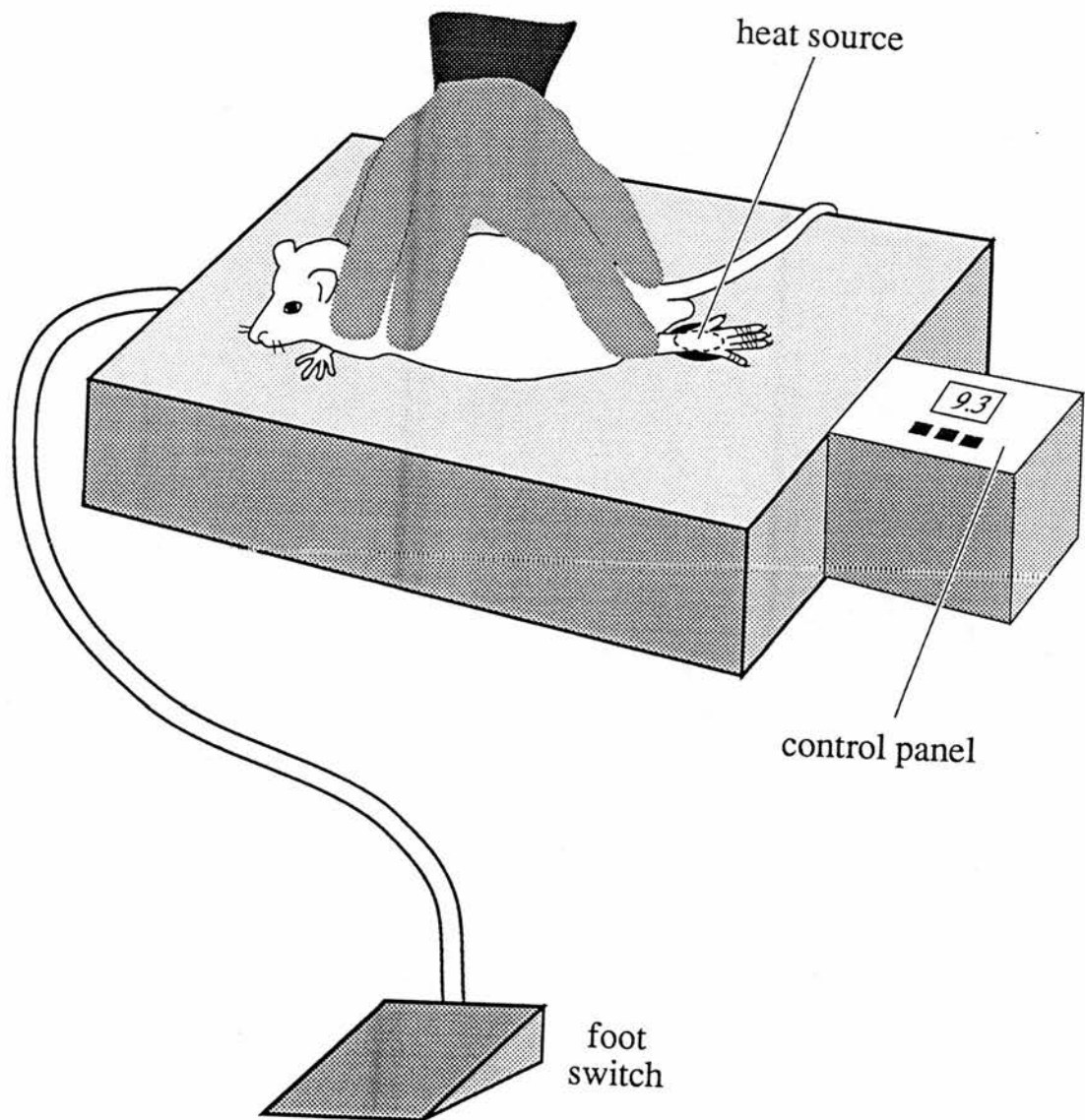
When the animal attempted to withdraw its paw the foot switch was released and the paw quickly removed.



## **Figure 5.2**

### **Schematic representation of testing paw withdrawal latency to noxious thermal stimulation**

The dorsal hairy surface of the foot was placed over a 50W infra-red bulb. Depression of a foot switch turned on the heat source (and the timer simultaneously), which was terminated when a circuit, relying on reflection of the foot, was broken. Withdrawal latencies were recorded to the nearest 0.1 second. The control panel allowed resetting of the display and manual operation of the heat source, if this was preferred. A cut-off latency of 23.0 seconds was automatic.



### **5.3. RESULTS**

Nociceptive reflex responses of groups of rats ( $6 \leq n \leq 16$ ) were assessed by their withdrawal reaction to a noxious (mechanical or thermal) stimulus applied to one hindpaw prior to and following intrathecal drug administration. The effects of drugs were compared in normal rats and those where an intraplantar injection of carrageenan had been given several (3 to 4) hours earlier, to cause inflammation to develop in one hindpaw. Withdrawal thresholds/latencies were calculated for each individual animal, and subsequently converted to a percentage of the reaction immediately prior to drug administration. The mean changes in paw withdrawal responses per group of rats are given (mean percentage change  $\pm$  SEM) with statistical significance as determined using the Mann-Whitney U-test.

Results are presented according to the stimulus given (noxious mechanical or thermal) and the state of the tested paw (non-inflamed or inflamed) and summarised in tables 5.1. - 5.4.

No non-nociceptive or motor testing, eg Rotarod, was carried out. On only one or two occasions did animals exhibit motor effects, ie paralysis or leg splaying, and were subsequently withdrawn from further testing. The data obtained from these animals was not used. The effects of habituation to noxious stimuli could not be monitored due to the very short time of the whole protocol (3 tests in a total of 5 minutes following the intrathecal injection).

#### **5.3.1. METABOTROPIC GLUTAMATE RECEPTOR ANTAGONISTS**

The effect of intrathecally administered metabotropic glutamate receptor antagonists, L-AP3 and (S)-CHPG, was assessed in animals with non-inflamed paws and with a unilateral carrageenan-induced inflammation. Paw withdrawal thresholds/latencies to noxious mechanical and thermal stimuli were measured before and after drugs were given. The effects on the two models were compared. D-AP3, the stereoisomer of L-AP3 with much reduced activity at mGlu receptors, was also investigated.

Comparisons of the effects of individual drugs on responses originating from non-inflamed and inflamed paws can be viewed graphically in figures 5.3.(a and b) and 5.5. The differential effects induced by the AP3 isomers on responses originating from carrageenan-treated paw can be seen in figure 5.4.

### **5.3.1.1. VEHICLE CONTROLS**

All drug-induced effects were compared to those of vehicle for analysis of statistical significance. In the case of mGlu receptor antagonists the vehicle was 0.9% saline.

#### **(a) Non-Inflamed Paw**

When 10 $\mu$ l of saline was injected intrathecally (n=12) there was a slight elevation in both mechanical and thermal responsiveness at 1 minute ( $132\pm 27\%$  and  $110\pm 5\%$  respectively, of  $3.9\pm 0.5$  units and  $10.4\pm 0.4$  seconds immediately prior to drug injection). This transient tendency to an increase in threshold/latency quickly subsided and by the third test, at 5 minutes, responses had returned very close to baseline ( $102\pm 11\%$  and  $102\pm 8\%$ ).

#### **(b) Inflamed Paw**

In a separate group of animals with carrageenan-induced inflammation of the ipsilateral hindpaw, saline caused a rise in thermal and mechanical thresholds (n=14) similar to those results obtained in the above group. However, in this case not all responses had returned to pre-drug controls ( $2.5\pm 0.3$  units and  $7.0\pm 0.5$  seconds) by the end of the tests. Although the response to noxious thermal stimulation was  $118\pm 7\%$  after 1 minute and  $112\pm 7\%$  at 5 minutes, withdrawal thresholds to noxious mechanical stimulation were  $141\pm 21\%$  and  $154\pm 40\%$  respectively.

### **5.3.1.2. THE EFFECT OF L- AND D-AP3 ON BEHAVIOURAL NOCICEPTIVE REFLEXES**

In summary, L-AP3 did not alter responses originating from the normal paw. However, following the induction of inflammation, due to carrageenan, this compound significantly increased paw withdrawal threshold/latency to both noxious mechanical and thermal stimulation (see figures 5.3.(a) and (b)). D-AP3 was without effect in all tests and produced responses significantly different from L-AP3 when assessing responses originating from the inflamed paw (see figure 5.4.).

Two doses of these compounds were tested, based on a previous study using them in such behavioural testing (Coderre & Melzack, 1992). However, when the tests were carried out both doses (30 and 60nmol actually injected; 2.5 and 5mg/ml stock) gave very similar results. As time did not permit the further construction of an adequate dose response curve, only the results from the higher dose will be discussed.

## **L-AP3**

### **(a) Non-inflamed Paw**

*Mechanical Nociceptive Reflex:* The intrathecal administration of L-AP3 failed to significantly alter paw withdrawal thresholds to noxious mechanical stimulation (with respect to saline controls,  $p > 0.05$ , Mann-Whitney U-test). One minute after the injection paw withdrawal thresholds were  $137 \pm 11\%$  of pre-drug  $3.7 \pm 0.2$  units, at 3 minutes they were  $132 \pm 11\%$  and by 5 minutes they had lowered to  $119 \pm 9\%$  ( $n=14$ ).

*Thermal Nociceptive Reflex:* Changes in the latencies for withdrawals to a noxious thermal stimulus matched those for mechanical stimulation. The shift in paw withdrawal latency was again insignificant ( $p > 0.05$ , Mann-Whitney U-test), rising to  $127 \pm 6\%$ ,  $117 \pm 8\%$  and  $105 \pm 4\%$  at 1, 3 and 5 minutes respectively of the pre-drug  $10.5 \pm 0.4$  seconds ( $n=14$ ).

### **(b) Inflamed Paw**

*Mechanical Nociceptive Reflex:* In contrast to its lack of effect on responses from the untreated paw, L-AP3 produced a significant rise, compared to saline, in paw withdrawal threshold following the induction of inflammation ( $n=14$ ). It increased the threshold by greater than two-fold ( $256 \pm 58\%$  of control ( $2.5 \pm 0.3$  units);  $p < 0.05$ , Mann Whitney U-test) at 3 minutes (see figure 5.3.(a)). This was the largest elevation in paw withdrawal threshold throughout the whole study. When the responses between the two groups (normal and carrageenan-treated) were compared, the results at 3 minutes post-drug showed a significant difference ( $p < 0.05$ , Mann Whitney U-test).

*Thermal Nociceptive Reflex:* Similar to the results with noxious mechanical stimuli, the resultant effect of L-AP3 was a significant shift in the latency ( $n=14$ ). This occurred at 1 minute, when the paw withdrawal latency was  $197 \pm 6\%$  of



the pre-drug  $6.9 \pm 0.4$  second control ( $p < 0.01$ , Mann Whitney U-test). The rise was still evident at 3 minutes ( $159 \pm 10\%$ ), but no longer significantly different from saline (see figure 5.3.(a)). The difference between latencies in normal and inflamed paws was significant at both 1 and 3 minutes ( $p < 0.01$ , Mann Whitney U-test).

Figure 5.3.(b) shows the significant difference in effect of L-AP3 on the responses of the inflamed paw, compared to those responses from the non-inflamed paw.

## **D-AP3**

### **(a) Non-inflamed Paw**

*Mechanical Nociceptive Reflex:* An intrathecal injection of D-AP3 ( $n=8$ ) did not significantly alter the paw withdrawal thresholds. After 1 minute the threshold had risen slightly to  $134 \pm 25\%$  of the pre-drug level of  $3.4 \pm 0.3$  units, and by 5 minutes was  $96 \pm 7\%$ ; similar to the observations following a saline injection.

*Thermal Nociceptive Reflex:* As with the responses to noxious mechanical stimulation, D-AP3 failed to change withdrawal latencies to thermal stimulation ( $n=8$ ). At 1 minute the latency was  $117 \pm 8\%$  of pre-drug control (which was  $10.9 \pm 0.5$  seconds) and by 5 minutes it was  $103 \pm 4\%$ .

### **(b) Inflamed Paw**

*Mechanical Nociceptive Reflex:* The response in the inflamed paw to intrathecal D-AP3 was no different from that of saline. Although the paw withdrawal thresholds were slightly higher at 1 and 3 minutes ( $160 \pm 27\%$  and  $171 \pm 24\%$  of pre-drug  $1.3 \pm 0.1$  units) this was not statistically significant from saline ( $p > 0.05$ , Mann Whitney U-test). However, at 3 minutes these values were significantly lower than the thresholds induced by intrathecal L-AP3 ( $p < 0.05$ , Mann Whitney U-test; see figure 5.4.).

*Thermal Nociceptive Reflex:* D-AP3 did not alter latencies to values significantly different from those in the presence of saline. At 1 minute the paw withdrawal latency was  $102 \pm 8\%$  of the pre-drug latency of  $7.0 \pm 0.4$  seconds and by 5 minutes it was  $94 \pm 5\%$  of this control. The latency at 1 minute was significantly lower than that seen when L-AP3 was injected ( $p < 0.05$ , Mann Whitney U-test; see figure 5.4.).

### 5.3.1.3. THE EFFECT OF (S)-CHPG ON BEHAVIOURAL REFLEXES

To summarise the results, (S)-CHPG significantly raised paw withdrawal thresholds/latencies to noxious mechanical and thermal stimuli. This occurred with responses originating from the normal paw and, in a separate group of animals, the paw with carrageenan-induced inflammation. See figure 5.5.

Two doses of this compound were tested, based on previous studies using L-AP3 in such behavioural testing. However, when the tests were carried out both doses (24 and 48nmol actually injected; 2.5 and 5mg/ml stock) gave very similar results, as with L-AP3. As time did not permit the further construction of an adequate dose response curve, only the results from the higher dose will be discussed.

#### (a) Non-inflamed Paw

*Mechanical Nociceptive Reflex:* The only significant change brought about in responses to a mechanical stimulus in the non-inflamed paw was by injection of (S)-CHPG (n=14). This occurred at 3 minutes, when paw withdrawal threshold rose to  $149 \pm 10\%$  of the pre-drug control level of  $3.8 \pm 0.2$  units ( $p < 0.05$ ).

*Thermal Nociceptive Reflex:* Again (S)-CHPG caused a significant shift in latencies at 3 minutes ( $p < 0.05$ , Mann Whitney U-test; n=14), with respect to saline controls. The mean withdrawal latency at 3 minutes was  $140 \pm 8\%$  of the pre-drug  $9.4 \pm 0.3$  seconds, and by 5 minutes was  $126 \pm 6\%$ .

#### (b) Inflamed Paw

*Mechanical Nociceptive Reflex:* Similar to the results observed when L-AP3 was injected, intrathecal (S)-CHPG brought about a significant rise in thresholds (n=13). After 1 minute paw withdrawal threshold had risen to  $230 \pm 31\%$  of the pre-drug control of  $2.9 \pm 0.3$  units ( $p < 0.05$ , with respect to saline). When the test was carried out at 3 minutes the mean threshold was  $193 \pm 32\%$ , but this did not reach significance. The increase observed at 1 minute was also significantly different from the same test in the non-inflamed foot ( $p < 0.01$ ).

*Thermal Nociceptive Reflex:* This reflex was significantly depressed by intrathecal (S)-CHPG at 1 minute (compared to saline controls,  $p < 0.01$ , Mann Whitney U-test; n=13), when the mean latency was  $174 \pm 10\%$  of pre-drug levels (8.0

$\pm 0.4$  seconds). As with the response to a mechanical stimulus, the withdrawal latency was still very high at 3 minutes,  $171 \pm 12\%$ , but no longer significantly different from responses observed with a saline injection ( $p > 0.05$ , Mann Whitney U-test).

### **5.3.2. TACHYKININ RECEPTOR ANTAGONISTS**

The effects of intrathecally administered tachykinin  $NK_1$  and  $NK_2$  receptor antagonists, GR82334 and L659,874 respectively, were assessed in separate groups of animals with non-inflamed paws and with a unilateral carrageenan-induced inflammation. Paw withdrawal responses to noxious mechanical and thermal stimuli were measured before and after drugs were given. The effects on the two models were compared.

Paw withdrawal thresholds/latencies were calculated for each individual animal, and subsequently converted to a percentage of the response immediately prior to drug administration. It is the mean changes in paw withdrawal thresholds/latencies per group which are given (mean percentage change  $\pm$  SEM). Statistical significance of differences from the effect of vehicle injection were determined using the Mann-Whitney U-test.

The results are divided according to the stimulus given (noxious mechanical or thermal) and the state of the paw (non-inflamed or inflamed) and are provided in summary form in tables 5.1. - 5.4. Comparisons of the effects of individual drugs on non-inflamed and inflamed paws is illustrated graphically in figures 5.6. and 5.7.

#### **5.3.2.1. VEHICLE CONTROLS**

Controls for the  $NK_1$  receptor antagonist GR82334 were saline, as described previously (section 5.3.1.1.). The  $NK_2$  receptor antagonist, L659,874 was administered in 0.3% dimethylsulphoxide (DMSO) in saline.

##### **(a) Non-Inflamed Paw**

When 10  $\mu$ l of DMSO in saline was injected intrathecally ( $n=6$ ) there was a slight elevation in both mechanical and thermal paw withdrawal response at 1

minute ( $111\pm12\%$  and  $112\pm12\%$  respectively), compared to pre-drug controls ( $4.8\pm0.1$  units and  $9.9\pm0.7$  seconds, defined as 100%). This small rise was seen until 5 minutes, when responses were  $108\pm12\%$  and  $115\pm11\%$  respectively. These changes in withdrawal threshold/latency are slightly less than those observed with intrathecally administered saline.

**(b) Inflamed Paw**

Similar to the results obtained in the above group, DMSO caused a nominal rise in thermal and mechanical paw withdrawal responses ( $n=14$ ). However, in this case some thresholds to noxious mechanical stimulation fell below pre-drug controls by the end of the tests (not markedly): at 1 minute the response to DMSO was  $120\pm11\%$  and at 5 minutes it was  $89\pm7\%$  of the pre-drug levels of  $2.9\pm0.2$  units. When the reflex to noxious thermal stimulation was tested the results were similar to those evoked by saline,  $107\pm5\%$  at 1 minute and  $115\pm8\%$  at 5 minutes of a pre-drug mean of  $6.8\pm0.5$  seconds.

**5.3.2.2. THE EFFECT OF NK<sub>1</sub> RECEPTOR ANTAGONIST GR82334 ON BEHAVIOURAL REFLEXES**

In summary, GR82334 failed to alter significantly any responses in the tests that were carried out in this investigation (see figure 5.6.).

Based on other behavioural analgesia studies carried out by the group, a dose of 3nmol (actual injection) was selected as a dose expected to be effective. This dose is above that previously shown to antagonise caudally-directed biting and scratching evoked by NK<sub>1</sub> receptor agonist substance P methyl ester (0.7 - 1nmol, Birch *et al*, 1991).

**(a) Non-inflamed Paw**

*Mechanical Nociceptive Reflex:* When GR82334 was administered, responses were not significantly altered during the ensuing 5 minutes ( $p>0.05$ , Mann Whitney U-test). After 1 minute paw withdrawal threshold was  $98\pm10\%$  of the pre-drug  $4.8\pm0.4$  units, and by 5 minutes was  $87\pm12\%$ .

*Thermal Nociceptive Reflex:* Latencies remained close to baseline measurements throughout the tests. At 1 minute mean latency was  $109\pm9\%$  and at 5 minutes  $113\pm9\%$ , compared to pre-drug values of  $9.9\pm0.7$  seconds.

**(b) Inflamed Paw**

*Mechanical Nociceptive Reflex:* As with the normal paw, the intrathecal injection of GR82334 failed to significantly change paw withdrawal thresholds ( $p>0.05$ , Mann Whitney U-test). Tests at 1 minute showed a mean response of  $126\pm 17\%$  (with respect to pre-drug  $2.8\pm 0.3$  units) and at 5 minutes it was very similar -  $127\pm 16\%$ .

*Thermal Nociceptive Reflex:* This test showed a similar trend to the others. No discernible changes in paw withdrawal latencies were brought about by GR82334. The mean latency at 1 minute was  $109\pm 6\%$  of the pre-drug  $7.9\pm 0.5$  seconds, and at 5 minutes was  $105\pm 6\%$  ( $p>0.05$ , Mann Whitney U-test).

**5.3.2.3. THE EFFECT OF NK<sub>2</sub> RECEPTOR ANTAGONIST L659,874 ON BEHAVIOURAL REFLEXES**

In summary, L659,874 selectively and significantly increased paw withdrawal responses to noxious thermal, but not mechanical, stimulation. This was the case in both the non-inflamed and the inflamed paw (see figure 5.7.).

As with GR82334, the choice of dose of L659,874 used in this study (3nmol) was based on data from other studies carried out by the group, in which this represented a maximally effective dose.

**(a) Non-inflamed Paw**

*Mechanical Nociceptive Reflex:* L659,874 did not significantly alter paw withdrawal thresholds to a mechanical stimulus ( $p>0.05$ , Mann Whitney U-test). One minute after the injection the mean threshold was  $107\pm 16\%$  compared to the mean pre-drug level of  $3.5\pm 0.5$  units, and by 5 minutes it was much the same,  $108\pm 13\%$ .

*Thermal Nociceptive Reflex:* This reflex was significantly depressed by the NK<sub>2</sub> receptor antagonist, for the duration of the test. At 1 minute paw withdrawal latency was  $183\pm 26\%$  of pre-drug  $7.7\pm 0.6$  seconds, at 3 minutes it was  $181\pm 20\%$  and finally at 5 minutes  $164\pm 13\%$  (all three values were  $p<0.01$ , Mann Whitney U-test).

**(b) Inflamed Paw**

*Mechanical Nociceptive Reflex:* There was no difference in the effect L659,874 had here, compared to the normal paw: it was still unsuccessful in changing paw withdrawal responses ( $p>0.05$ , Mann Whitney U-test). The mean threshold after 1 minute was  $126\pm16\%$  and at 5 minutes was  $115\pm18\%$  of pre-drug  $3.5\pm0.5$  units.

*Thermal Nociceptive Reflex:* The effect of L659,874 in the normal paw was mirrored in this test. Once again it induced significant rises in paw withdrawal latencies, although this time only in the first two tests. After 1 minute the mean latency was  $191\pm21\%$  and at 3 minutes was  $170\pm17\%$  (both  $p<0.01$ , with respect to pre-drug levels of  $6.7\pm0.6$ , Mann Whitney U-test). The withdrawal latency at 5 minutes ( $150\pm15\%$ ) was still elevated, but no longer statistically significant ( $p>0.05$ , Mann Whitney U-test).

### **5.3.3. THE EFFECT OF CO-INJECTION OF METABOTROPIC GLUTAMATE AND TACHYKININ RECEPTOR ANTAGONISTS ON BEHAVIOURAL NOCICEPTIVE REFLEXES**

Following the study on the effect of each individual compound on nociceptive reflexes, the effect of intrathecally co-administered metabotropic glutamate receptor antagonists, L-AP3 and (S)-CHPG, with either (1) NK<sub>1</sub> receptor antagonist GR82334 or (2) NK<sub>2</sub> receptor antagonist L659,874 was assessed in animals with non-inflamed paws and in a separate group with a unilateral carrageenan-induced inflammation. Paw withdrawal thresholds/latencies to noxious mechanical and thermal stimuli were measured before and after drugs were given. The effects on the two models were compared.

Paw withdrawal thresholds/latencies were calculated for each individual animal, and subsequently converted to a percentage of the response immediately prior to drug administration. Results for each group were then expressed as mean percentage change  $\pm$  SEM. Statistical significance was determined using the Mann-Whitney U-test.

The results are divided according to the stimulus given (noxious mechanical or noxious thermal) and the state of the paw (non-inflamed or inflamed) and are provided in summary form in tables 5.1. to 5.4. The effect of co-administering drugs can be compared to the individual components of the injection in figures 5.8-5.11.

#### **5.3.3.1. THE EFFECT OF MGLU RECEPTOR ANTAGONISTS, CO-INJECTED WITH NK<sub>1</sub> RECEPTOR ANTAGONIST GR82334, ON BEHAVIOURAL REFLEXES**

In summary, all significant reductions in paw withdrawal responses brought about by the sole injection of a metabotropic glutamate receptor (mGluR) antagonist were attenuated when GR82334 was simultaneously administered. GR82334 had no significant effect when injected alone (Figure 5.6). However, GR82334 reduced the mGluR antagonist-induced increases on paw withdrawal responses, although none of these decreases led to values significantly lower than those seen when saline was injected (see figures 5.8. and 5.10.).



**(a) Non-inflamed Paw**

*Mechanical Nociceptive Reflex:*

(i) L-AP3: L-AP3 did not alter this reflex when given alone, and co-injection with GR82334 resulted in no change. Mean threshold was  $112 \pm 14\%$  compared to pre-drug values of  $3.6 \pm 0.2$  units at 1 minute, and  $115 \pm 11\%$  by 5 minutes.

(ii) (S)-CHPG: When given on its own this compound significantly raised the threshold of response. However, following a co-injection with GR82334, at 1 minute the paw withdrawal threshold was  $107 \pm 13\%$  of a pre-drug level of  $4.1 \pm 0.3$  units, significantly lower than the effect of the mGluR antagonist alone ( $147 \pm 9\%$ ;  $p < 0.05$ , see figure 5.10.).

*Thermal Nociceptive Reflex*

(i) L-AP3: No significant changes in paw withdrawal latencies were seen either with L-AP3 alone or in combination with GR82334. There was little alteration from pre-drug controls ( $8.2 \pm 0.6$  seconds): one minute after the co-injection the mean latency was  $110 \pm 9\%$  and at 5 minutes was  $104 \pm 10\%$

(ii) (S)-CHPG Although withdrawal latencies were decreased due to the co-injection ( $120 \pm 12\%$  and  $131 \pm 12\%$  at 1 and 3 minutes respectively, of a pre-drug latency of  $9.6 \pm 0.7$  seconds), compared to that of (S)-CHPG alone ( $138 \pm 7\%$  and  $140 \pm 8\%$ ), the change was not statistically significant ( $p > 0.05$ , Mann Whitney U-test).

**(b) Inflamed Paw**

*Mechanical Nociceptive Reflex*

(i) L-AP3: Administration of L-AP3 alone produced significantly increased responses at 3 minutes post-injection. When L-AP3 was injected together with GR82334, thresholds at both 1 minute ( $119 \pm 8\%$  (of pre-drug  $3.4 \pm 0.2$  units) compared to  $223 \pm 47\%$ ;  $p < 0.05$ , Mann-Whitney U-test) and 3 minutes ( $111 \pm 8\%$  compared to  $256 \pm 58\%$ ;  $p < 0.01$ ) were significantly lower than with the mGluR antagonist alone (see figure 5.8.).

(ii) (S)-CHPG: Similar to the results with L-AP3, this compound increased paw withdrawal thresholds at 1 minute when given alone. However the co-injection with GR82334 prevented this facilitation - after 1 minute the co-injection resulted in a significantly lower mean threshold of  $154 \pm 19\%$  (of pre-drug  $2.5 \pm 0.3$  units), with (S)-CHPG alone  $230 \pm 31\%$  ( $p < 0.01$ , Mann-Whitney U-test). Also, at 3 minutes the difference between the two treatments was significant, (S)-CHPG



administration resulted in a rise to  $193 \pm 32\%$  of pre-drug controls, whereas the co-injection only increased to  $132 \pm 15\%$  ( $p < 0.05$ , Mann-Whitney U-test). This is presented in figure 5.10.

#### *Thermal Nociceptive Reflex*

(i) L-AP3: At both 1 and 3 minutes post-injection the difference between the effect of L-AP3 alone and its co-administration with GR82334 was significant ( $p < 0.01$ ). The 1 minute paw withdrawal latencies were  $197 \pm 6\%$  and  $109 \pm 9\%$ , of a pre-drug level of  $7.6 \pm 0.5$  seconds, respectively, and at 3 minutes they were  $159 \pm 10\%$  and  $115 \pm 9\%$ . (see figure 5.8.)

(ii) (S)-CHPG: All three time points showed significantly lowered latencies when (S)-CHPG was co-administered with GR82334, compared to when given alone ( $p < 0.01$ , Mann-Whitney U-test). Latencies after co-administration were  $111 \pm 7\%$ ,  $117 \pm 8\%$  and  $109 \pm 6\%$  or pre-drug controls of  $6.2 \pm 0.4$  seconds at 1, 3 and 5 minutes post-injection respectively, whereas the corresponding values for (S)-CHPG alone were  $174 \pm 10\%$ ,  $171 \pm 12\%$  and  $143 \pm 8\%$  (see figure 5.10).

#### **5.3.3.2. THE EFFECT OF MGLU RECEPTOR ANTAGONISTS, CO-INJECTED WITH NK<sub>2</sub> RECEPTOR ANTAGONIST L659,874, ON BEHAVIOURAL REFLEXES**

In summary, latencies to noxious thermal stimulation in the normal paw, which were previously increased by L659,874, were decreased when co-administered with either metabotropic glutamate receptor (mGluR) antagonist, even when (as in the case of (S)-CHPG) the mGluR antagonist alone also elevated withdrawal latency. Similar effects were seen in response to stimulation of inflamed paws (where both L-AP3 and (S)-CHPG, and also L659,874 were effective alone) although the inhibition of elevated latencies did not reach statistical significance. L659,874 alone did not increase noxious mechanical response thresholds in normal or inflamed paws, but did significantly reduce the increase in response due to (S)-CHPG in both cases and L-AP3 in the inflamed paw.

##### **(a) Non-inflamed Paw**

#### *Mechanical Nociceptive Reflex*

(i) L-AP3: Co-injection of L-AP3 with L659,874 produced paw withdrawal responses which were no different from saline or from either drug alone.

At 1 minute the mean threshold was  $117 \pm 11\%$  of a mean pre-drug control of  $3.8 \pm 0.2$  units, and at 5 minutes it was  $125 \pm 8\%$ .

(ii) (S)-CHPG: Although (S)-CHPG alone significantly increased paw withdrawal thresholds at 3 minutes post-injection ( $149 \pm 10\%$ ), the lowering of this with L659,874 co-administered ( $121 \pm 12\%$  of pre-drug  $3.6 \pm 0.3$  units) was not statistically different.

#### *Thermal Nociceptive Reflex*

(i) L-AP3: Alone L659,874 significantly depressed this reflex at all three time points (with latencies of  $183 \pm 26\%$ ,  $181 \pm 20\%$  and  $164 \pm 13\%$  of pre-drug controls at 1, 3 and 5 minutes respectively;  $p < 0.01$ , Mann-Whitney U-test). However, when given simultaneously with L-AP3 this was no longer the case. Latencies at 3 and 5 minutes were significantly lowered to  $135 \pm 5\%$  and  $108 \pm 6\%$  of a mean pre-drug level of  $9.5 \pm 0.4$  seconds (compared to L659,874 alone,  $p < 0.01$ , Mann-Whitney U-test). These values were not statistically different from those obtained with L-AP3 given on its own.

(ii) (S)-CHPG: The co-injection of (S)-CHPG with L659,874 produced shorter paw withdrawal latencies than the elevated levels seen with L659,874 alone. This reduction was significant only at 5 minutes post-injection ( $132 \pm 8\%$  compared to  $164 \pm 13\%$  of pre-drug control of  $8.6 \pm 0.7$  seconds;  $p < 0.05$ , Mann Whitney U-test)

### **(b) Inflamed Paw**

#### *Mechanical Nociceptive Reflex*

(i) L-AP3: The previously high threshold seen after 3 minutes with L-AP3 alone ( $256 \pm 58\%$  of pre-drug levels) was no longer evident when L659,874 was given at the same time ( $148 \pm 39\%$  of a pre-drug mean of  $2.9 \pm 0.3$  units;  $p < 0.05$ , Mann Whitney U-test), even though L659,874 had no effect alone.

(ii) (S)-CHPG: As with the L-AP3, the increase in threshold normally observed with this compound was reversed when the  $NK_2$  antagonist was co-injected. Instead of a paw withdrawal threshold of  $230 \pm 31\%$  of pre-drug control at 1 minute ((S)-CHPG alone), the response was reduced to  $155 \pm 21\%$  of pre-drug levels (which were  $2.9 \pm 0.2$  units) with (S)-CHPG/L659,874 combination ( $p < 0.05$ , Mann Whitney U-test).

#### *Thermal Nociceptive Reflex*

(i) L-AP3: Both compounds alone and the co-injection significantly increased latencies. There was no statistically significant difference between the

effect of the co-injection ( $194\pm 20\%$ ,  $186\pm 14\%$  and  $129\pm 11\%$  of pre-drug values of  $5.9\pm 0.3$  seconds at 1, 3 and 5 minutes post-injection, respectively) and either of its components..

(ii) (S)-CHPG: All three tests following the co-injection showed lower latencies than with the individual components, although the differences were not statistically significant ( $p>0.05$ , Mann Whitney U-test).

**Table 5.1.**

**A summary of the changes brought about in paw withdrawal responses by  
intrathecal drug administration**

A brief summary of statistically significant increases and decreases in withdrawal responses induced by intrathecally administered metabotropic glutamate (mGlu) and neurokinin (NK) receptor antagonists, as determined by the Mann Whitney U-test ( $p < 0.05$ ).

mGlu receptor antagonists	L-AP3 and (S)-CHPG
NK <sub>1</sub> receptor antagonist	GR82334
NK <sub>2</sub> receptor antagonist	L659,874

	MECHANICAL NOCICEPTIVE REFLEX	THERMAL NOCICEPTIVE REFLEX
NON-INFLAMED PAW	<p>↑ with (S)-CHPG</p> <p>(S)-CHPG-induced ↑ is ↓ when co-injected with GR82334</p>	<p>↑ with (S)-CHPG and L659,874</p> <p>L659,874-induced ↑ is ↓ is when co-injected with L-AP3 or (S)-CHPG</p>
INFLAMED PAW	<p>↑ with L-AP3 and (S)-CHPG</p> <p>both mGluR antagonist-induced ↑ are ↓ when co-injected with GR82334 or L659,874</p>	<p>↑ with L-AP3, (S)-CHPG and L659,874</p> <p>both mGluR antagonist-induced ↑ are ↓ when co-injected with GR82334</p>

**Table 5.2**

**A summary of the changes brought about in paw withdrawal thresholds to noxious *mechanical* stimulation in the *non-inflamed* foot, by metabotropic glutamate and tachykinin receptor antagonists.**

These results are also presented in figures 5.3. - 5.11.

The effect of the following antagonists on paw withdrawal thresholds was investigated: L-AP3 (along with its isomer D-AP3) and (S)-CHPG (metabotropic glutamate receptors {mGluRs}), GR82334 (NK<sub>1</sub> receptor) and L659, 874 (NK<sub>2</sub> receptor).

The third column is the mean $\pm$ SEM of raw data for thresholds immediately preceding the intrathecal injection. This was considered as 100%. From raw data for each animal individual changes in threshold were calculated as a percentage, then the mean for each group was determined.

Statistical significance of changes relative to vehicle controls and co-injection of compounds were determined using the Mann-Whitney U-test.

The mean paw withdrawal threshold prior to drug administration was 4.0 $\pm$ 0.9 units<sup>#</sup> (n=97) though percentage changes were calculated, and are shown, per group.

All compounds, singularly or as a co-injection, with the sole exception of (S)-CHPG, failed to significantly alter withdrawal thresholds to a greater extent than vehicle. When (S)-CHPG was co-administered with GR82334 the resultant effect was a significantly lower withdrawal latency compared to that of the metabotropic glutamate receptor antagonist alone.

<sup>#</sup> units are described in section 5.2.4.1.

\*\* = p<0.01, \* = p<0.05, with respect to saline

■ ■ = p<0.01, ■ = p<0.05, with respect to (S)-CHPG

Drug Treatment	n	Threshold units at t=0	Threshold as % of that immediately prior to drug injection			
			1 min	3 mins	5 mins	
saline	12	3.9±0.5	132±27	116±14	102±11	
DMSO, 0.3%	6	4.8±0.1	111±12	101±6	108±12	
L-AP3, 60nmol	14	3.7±0.2	137±11	132±11	119±9	
D-AP3, 60nmol	8	3.4±0.3	134±25	94±7	96±7	
(S)-CHPG, 48nmol	14	3.8±0.2	147±9	149±10*	117±7	
GR82334, 3nmol	6	4.8±0.4	98±10	97±7	87±12	
L659, 874, 3nmol	6	3.5±0.5	107±16	97±2	108±13	
GR82334 + L-AP3	7	3.6±0.2	112±14	131±18	115±11	
L659,874 + L-AP3	8	3.8±0.2	117±11	116±14	125±8	
GR82334 + (S)-CHPG	8	4.1±0.3	107±13■	142±16	120±11	
L659,874 + (S)-CHPG	8	3.6±0.3	132±30	121±12	109±9	

**Table 5.3.**

**A summary of the changes brought about in paw withdrawal latencies to noxious *thermal* stimulation in the *non-inflamed* foot, by metabotropic glutamate and tachykinin receptor antagonists.**

These results are also presented in figures 5.3. - 5.11.

The effect of the following antagonists on paw withdrawal latencies was investigated: L-AP3 (along with its isomer D-AP3) and (S)-CHPG (metabotropic glutamate receptors {mGluRs}), GR82334 (NK<sub>1</sub> receptor) and L659, 874 (NK<sub>2</sub> receptor).

The third column is the mean $\pm$ SEM of raw data for latencies immediately preceding the intrathecal injection. This was considered as 100%. From raw data for each animal individual changes in latency were calculated as a percentage, then the mean for each group was determined.

Statistical significance of changes relative to vehicle controls and co-injection of compounds were determined using the Mann-Whitney U-test.

Mean pre-drug paw withdrawal latency was 9.4 $\pm$ 0.3 secs, n=97.

As with the response to noxious mechanical stimulation, (S)-CHPG produced a significant change in paw withdrawal latency, both from saline and a co-injection with GR82334. However, the most striking result in this study was the selective elevation in withdrawal latency to a noxious thermal induced by NK<sub>2</sub> receptor antagonist L659,874. This compound produced an increase in latencies at all three time points, all p<0.01.

\*\* = p<0.01, \* = p<0.05, with respect to saline

◆◆ = p<0.01, ◆ = p<0.05, with respect to L659,874



Drug Treatment	n	Latency at t=0 (secs)	Latency as a % of that immediately prior to drug injection			
			1 min	3 mins	5 mins	
saline	12	10.4±0.4	110±5	92±5	102±8	
DMSO, 0.3%	6	9.9±0.7	112±12	120±10	115±11	
L-AP3, 60nmol	14	10.5±0.4	127±6	117±8	105±4	
D-AP3, 60nmol	8	10.9±0.5	117±8	98±4	103±4	
(S)-CHPG, 48nmol	14	9.4±0.3	138±7	140±8*	126±6	
GR82334, 3nmol	6	8.6±0.9	109±9	119±6	113±9	
L659, 874, 3nmol	6	7.7±0.6	183±26**	181±20**	164±13**	
GR82334 + L-AP3	7	8.2±0.6	110±9	114±7	104±10	
L659,874 + L-AP3	8	9.5±0.4	141±6	135±5♦♦	108±6♦♦	
GR82334 + (S)-CHPG	8	9.6±0.7	120±12	131±12	114±9	
L659,874 + (S)-CHPG	8	8.6±0.7	153±15	137±10	132±8♦	

**Table 5.4.**

**A summary of the changes brought about in paw withdrawal thresholds to noxious *mechanical* stimulation in the *inflamed* foot, by metabotropic glutamate and tachykinin receptor antagonists.**

These results are also presented in figures 5.3. - 5.11.

As in Tables 5.1. and 5.2., the effect of the following antagonists on paw withdrawal thresholds was investigated: L-AP3 (along with its isomer D-AP3) and (S)-CHPG (metabotropic glutamate receptors {mGluRs}), GR82334 (NK<sub>1</sub> receptor) and L659, 874 (NK<sub>2</sub> receptor). These tables however show the drug-induced changes 3 hours after a unilateral injection of lambda carrageenan into the ipsilateral hindpaw.

The third column is the mean $\pm$ SEM of raw data for thresholds immediately preceding the intrathecal injection. This was considered as 100%. From raw data for each animal individual changes in threshold were calculated as a percentage, then the mean for each group was determined.

Statistical significance of changes relative to vehicle controls and co-injection of compounds were determined using the Mann-Whitney U-test.

Mean pre-drug thresholds were  $2.7\pm 0.2$  units<sup>#</sup> (n=155). This represents the carrageenan-induced reduction, of which the average was  $65\pm 26\%$  (n=155).

Both metabotropic glutamate receptor (mGluR) antagonists produced a significant shift in latencies from saline ( $p<0.05$ ), whereas in the non-inflamed state only (S)-CHPG did so. Also, the effect observed due to their co-administration with GR82334 was significantly reduced from the effect each mGluR antagonist had alone ( $p<0.01$ ).

<sup>#</sup>units are described in section 5.2.4.1.

\*\* =  $p<0.01$ , \* =  $p<0.05$ , with respect to saline

•• =  $p<0.01$ , • =  $p<0.05$ , with respect to L-AP3

■■ =  $p<0.01$ , ■ =  $p<0.05$ , with respect to (S)-CHPG

Drug Treatment	n	Threshold units at t=0	Threshold as a % of that immediately prior to drug injection		
			1 min	3 mins	5 mins
saline	14	2.5±0.3	141±21	141±21	154±40
DMSO, 0.3%	14	2.9±0.2	120±11	93±8	89±7
L-AP3, 60nmol	14	2.5±0.3	223±47	256±58*	159±28
D-AP3, 60nmol	8	1.3±0.1	160±27	171±24	125±16
(S)-CHPG, 48nmol	13	2.9±0.3	230±31*	193±32	164±29
GR82334, 3nmol	14	2.8±0.3	126±17	140±22	127±16
L659, 874, 3nmol	14	2.7±0.3	126±16	125±22	115±18
GR82334 + L-AP3	16	3.4±0.2	119±8•	111±8••	110±8
L659,874 + L-AP3	16	2.9±0.3	189±43	148±39•	109±16
GR82334 + (S)-CHPG	16	2.5±0.3	154±19■■	132±15■	109±8
L659,874 + (S)-CHPG	16	2.9±0.2	155±21■	146±19	131±14

**Table 5.5.**

**A summary of the changes brought about in paw withdrawal latencies to noxious *thermal* stimulation in the *inflamed* foot, by metabotropic glutamate and tachykinin receptor antagonists.**

These results are also presented in figures 5.3. - 5.11.

As in Tables 5.1. and 5.2., the effect of the following antagonists on paw withdrawal latencies was investigated: L-AP3 (along with its isomer D-AP3) and (S)-CHPG (metabotropic glutamate receptors {mGluRs}), GR82334 (NK<sub>1</sub> receptor) and L659, 874 (NK<sub>2</sub> receptor). These tables however show the drug-induced changes 3 hours after a unilateral injection of lambda carrageenan into the ipsilateral hindpaw.

The third column is the mean±SEM of raw data for latencies immediately preceding the intrathecal injection. This was considered as 100%. From raw data for each animal individual changes in latency were calculated as a percentage, then the mean for each group was determined.

Statistical significance of changes relative to vehicle controls and co-injection of compounds were determined using the Mann-Whitney U-test.

The mean latency immediately prior to drug administration was 7.0±0.2 secs. This represents the carrageenan-induced reduction, of which the average was 38±12% (n=155). Similar to the mechanical results, the mGluR antagonists caused a significant rise in withdrawal latencies from both saline and the co-injection with GR82334. Once again L659,874 induced the most significant rise in latency.

\*\* = p<0.01, \* = p<0.05, with respect to saline

•• = p<0.01, • = p<0.05, with respect to L-AP3

■■ = p<0.01, ■ = p<0.05, with respect to (S)-CHPG

Drug Treatment	n	Latency at t=0 (secs)	Latency as a % of that immediately prior to drug injection		
			1 min	3 min	5 min
saline	14	7.0±0.5	118±7	129±9	112±7
DMSO, 0.3%	14	6.8±0.5	107±5	113±8	115±8
L-AP3, 60nmol	14	6.9±0.4	197±6**	159±10	132±10
D-AP3, 60nmol	8	7.0±0.4	102±8	97±7	94±5
(S)-CHPG, 48nmol	13	8.0±0.4	174±10*	171±12	143±8
GR82334, 3nmol	14	7.9±0.5	109±6	105±7	97±3
L659, 874, 3nmol	14	6.7±0.6	191±21**	170±17**	150±15
GR82334 + L-AP3	16	7.6±0.5	109±9●●	115±9●●	105±6
L659,874 + L-AP3	16	5.9±0.3	194±20	186±14	129±11
GR82334 + (S)-CHPG	16	6.2±0.4	111±7■■■	117±8■■■	109±6■■■
L659,874 + (S)-CHPG	16	7.4±0.5	169±14	142±9	120±8

**Figure 5.3.(a)**

**The effect of intrathecal injection of L-AP3 on paw withdrawal responses in the non-inflamed and inflamed paws**

The mean response to noxious mechanical and thermal stimulation at time 0 in the non-inflamed group were  $3.8 \pm 0.1$  units<sup>#</sup> and  $10.5 \pm 0.5$  seconds respectively (n=28).

Metabotropic glutamate receptor antagonist L-AP3 failed to alter these thresholds/latencies (n=14). However, when tests were repeated in a second group of animals 3 hours after a unilateral injection of carrageenan into the ipsilateral hindpaw (mean pre-drug responses in this group =  $2.5 \pm 0.3$  units and  $7.0 \pm 0.1$  secs; mean reductions by carrageenan were  $109 \pm 9\%$  and  $44 \pm 6\%$ , n=28), L-AP3 caused statistically significant increases in response threshold in both tests.

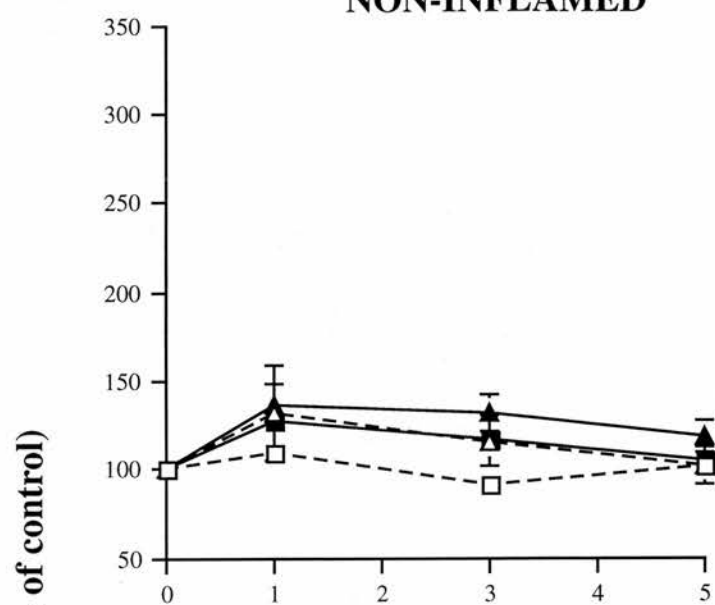
Statistical significance was evaluated using the Mann-Whitney U-test.

\*\* indicates  $p < 0.01$ , and \* is  $p < 0.05$ , with respect to saline controls.

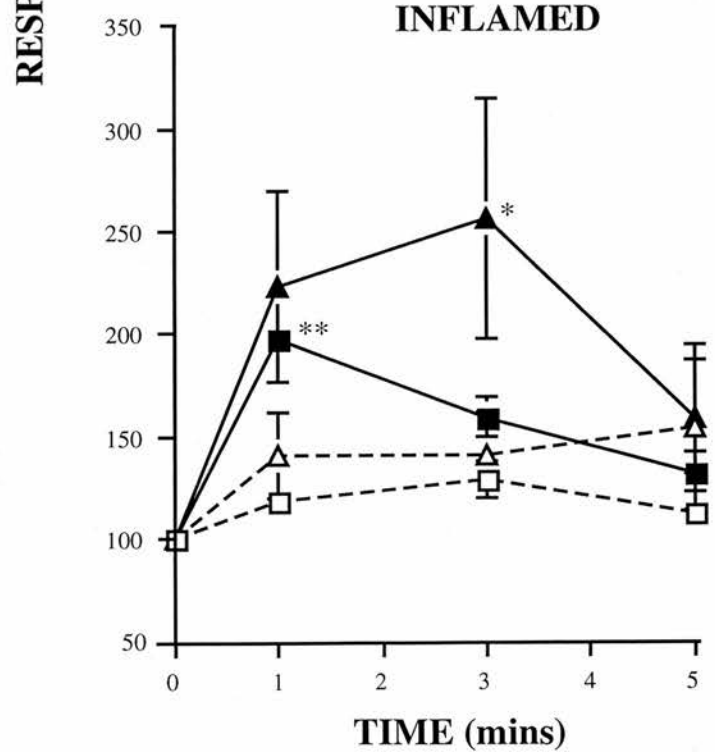
# units are described in section 5.2.4.1.

- △-- saline MECHANICAL threshold
- saline THERMAL latency
- ▲- L-AP3, 60nmol MECHANICAL threshold
- L-AP3, 60nmol THERMAL latency

# NON-INFLAMED



# INFLAMED



**Figure 5.3.(b)**

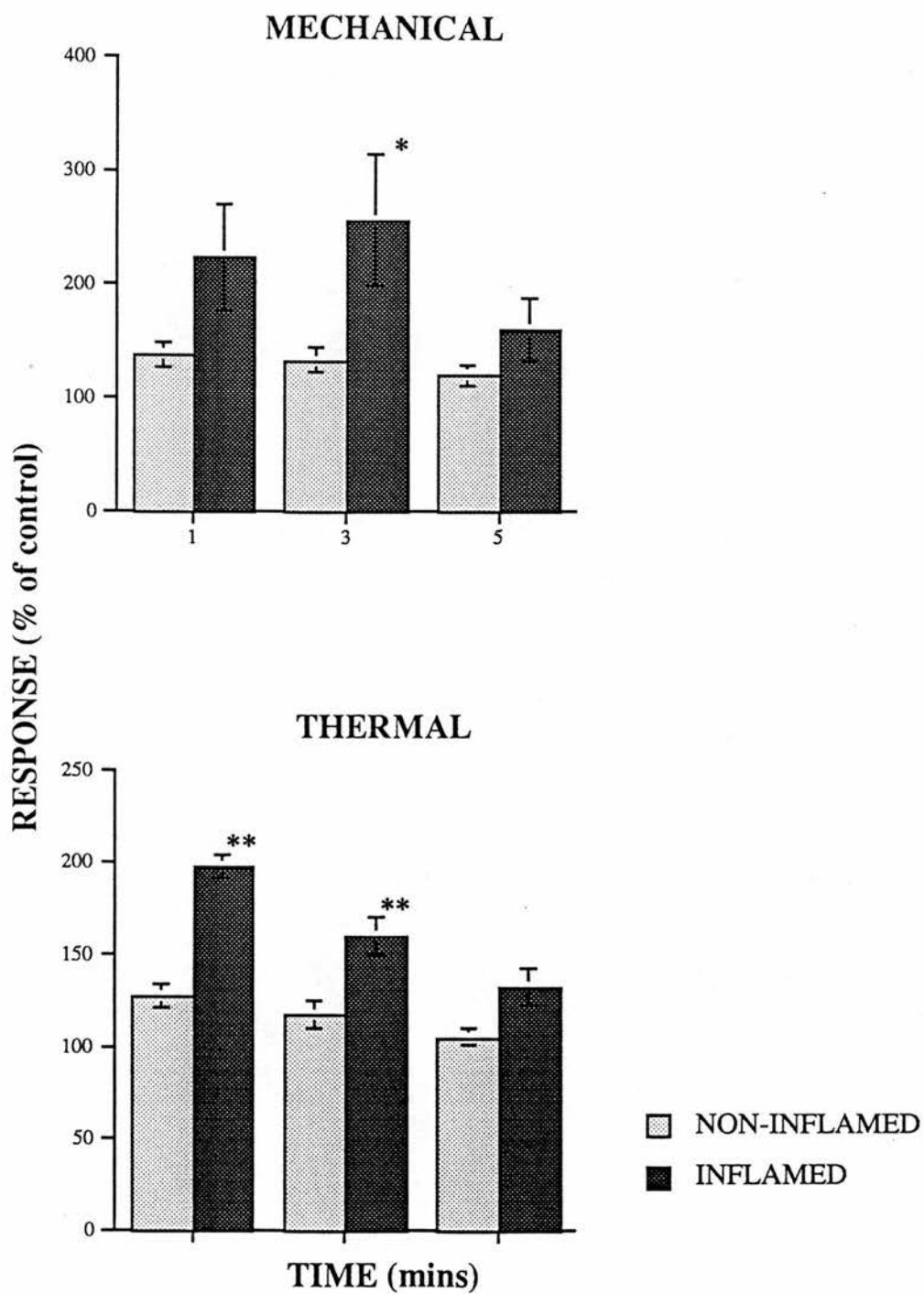
**Comparison of the effect of intrathecal injection of L-AP3 on paw withdrawal responses in the non-inflamed and inflamed paws**

The histograms show the effect of L-AP3 on responses originating from non-inflamed and inflamed paws, as a percentage of pre-drug controls. This compound was significantly more effective in raising withdrawal responses to noxious mechanical and noxious thermal stimulation in the carrageenan-inflamed paw, with respect to the untreated paw.

Statistical significance was evaluated using the Mann-Whitney U-test.

\*\* =  $p < 0.01$ ; \* =  $p < 0.05$ , with respect to the non-inflamed paw.





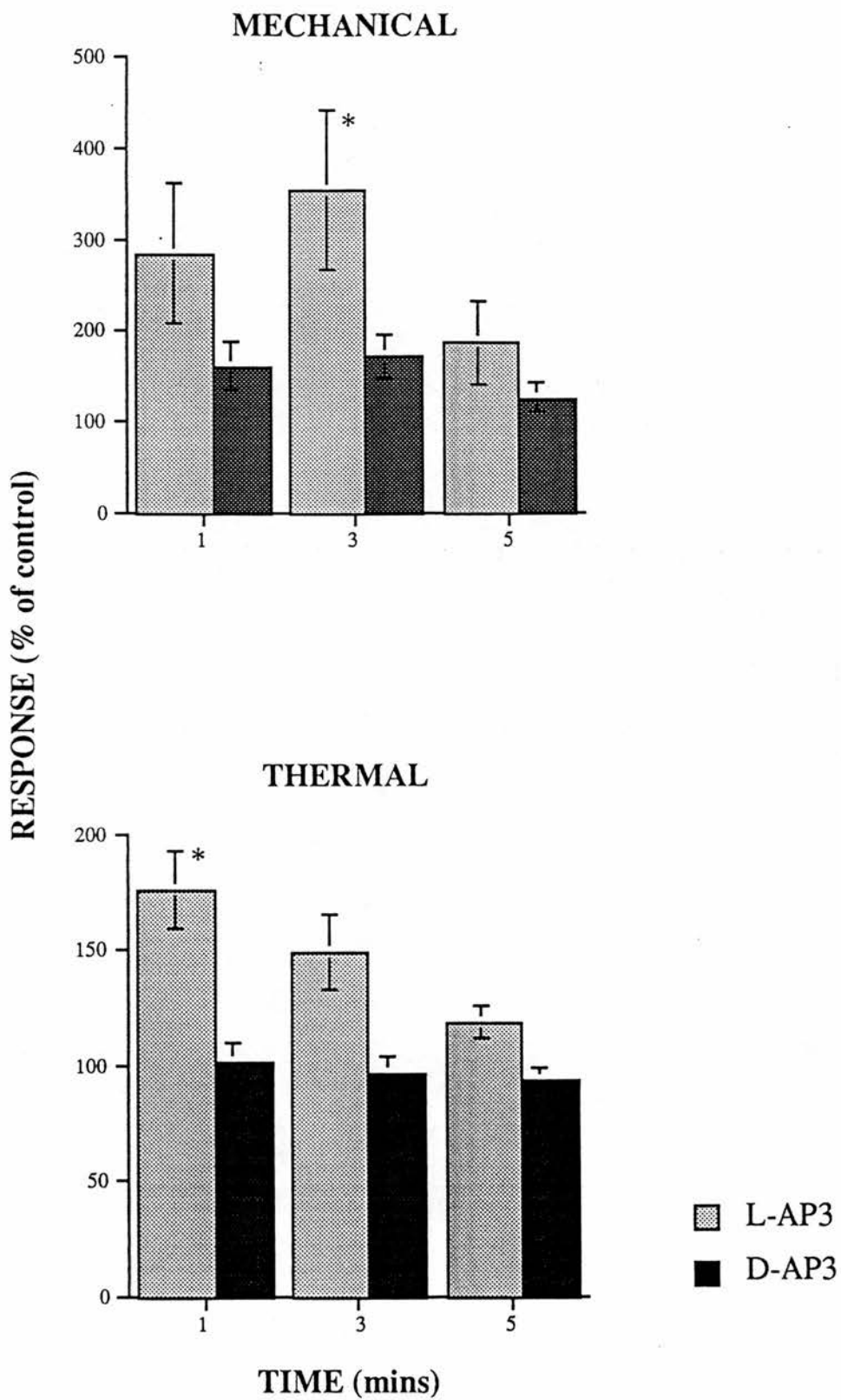
**Figure 5.4.**

**Comparison of the effects of intrathecal injection of L-AP3 and its isomer D-AP3 on paw withdrawal responses in the inflamed paw**

The histograms show a comparison of the effects each of the AP3 isomers had on paw withdrawal responses, when intrathecally injected (60nmol). L-AP3, the isomer believed to block metabotropic glutamate receptors, induced a significant rise in responses to both noxious mechanical and thermal stimulation. D-AP3 produced results similar to those of a saline injection. Responses in the presence of L-AP3 were significantly greater than those with D-AP3.

Statistical significance was evaluated using the Mann-Whitney U-test.

\* indicates  $p < 0.05$ , with respect to D-AP3.



### Figure 5.5

#### The effect of intrathecal injection of (S)-CHPG on paw withdrawal responses in the non-inflamed and inflamed paws

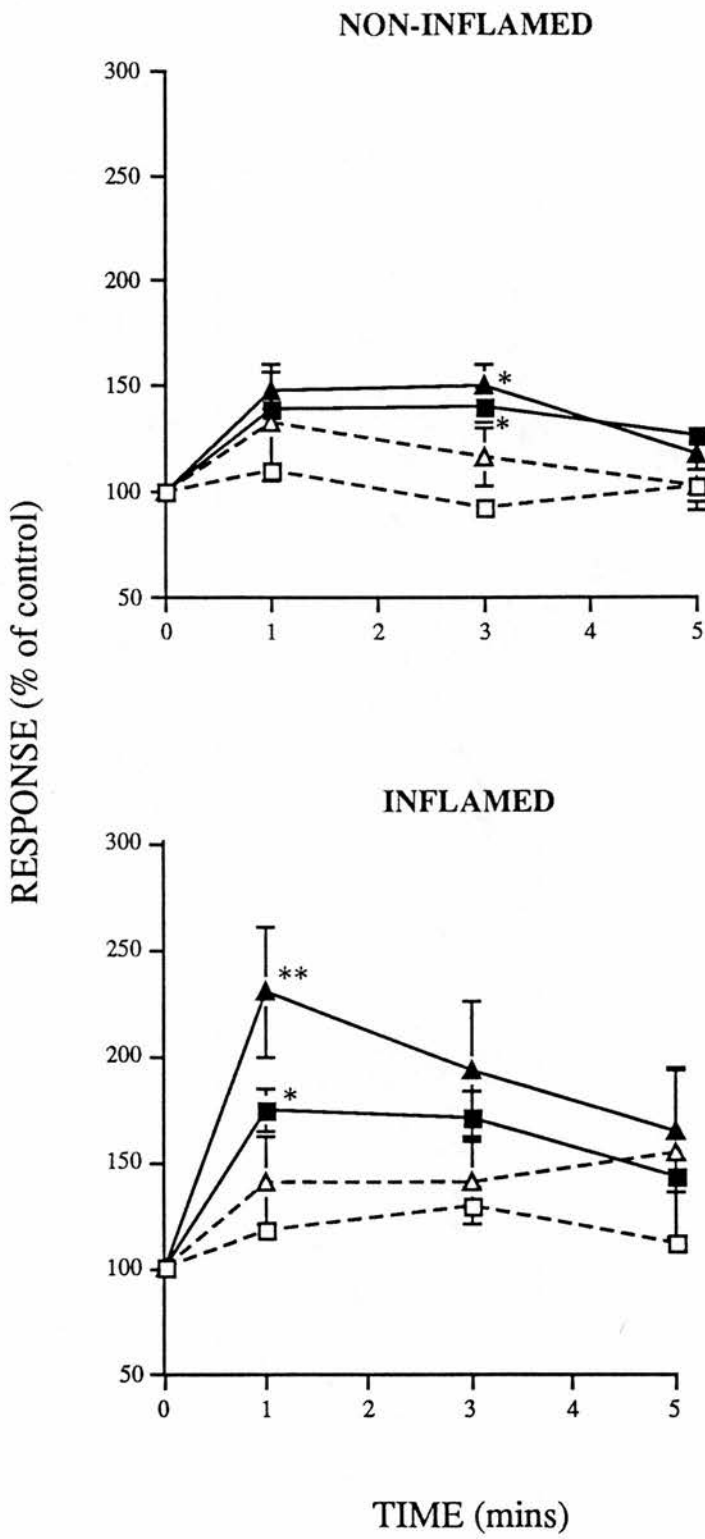
The mean thresholds from noxious mechanical and latencies from noxious thermal stimulation at time 0 in the non-inflamed group were  $3.9 \pm 0.1$  units<sup>#</sup> and  $9.5 \pm 0.5$  seconds respectively (n=26). In a separate group of animals with inflamed paws the pre-drug responses were  $2.7 \pm 0.2$  units and  $7.5 \pm 0.5$  seconds (carrageenan-induced reductions of  $79 \pm 21\%$  and  $39 \pm 1\%$ ; n=27).

Metabotropic glutamate receptor antagonist (S)-CHPG brought about a significant elevation in withdrawal responses, with respect to saline effects, when the paw received a noxious mechanical or thermal stimulation. This rise in threshold/latency was observed in both the non-inflamed state and following carrageenan-induced inflammation.

Significance was calculated using the Mann-Whitney U-test. \*\*= $p < 0.01$  and \*= $p < 0.05$  with respect to saline

#units are described in section 5.2.4.1.

- Δ-- saline MECHANICAL threshold
- saline THERMAL latency
- ▲- (S)-CHPG, 48nmol MECHANICAL threshold
- (S)-CHPG, 48nmol THERMAL latency



**Figure 5.6.**

**The effect of intrathecal injection of GR82334 on paw withdrawal responses  
in the non-inflamed and inflamed paws**

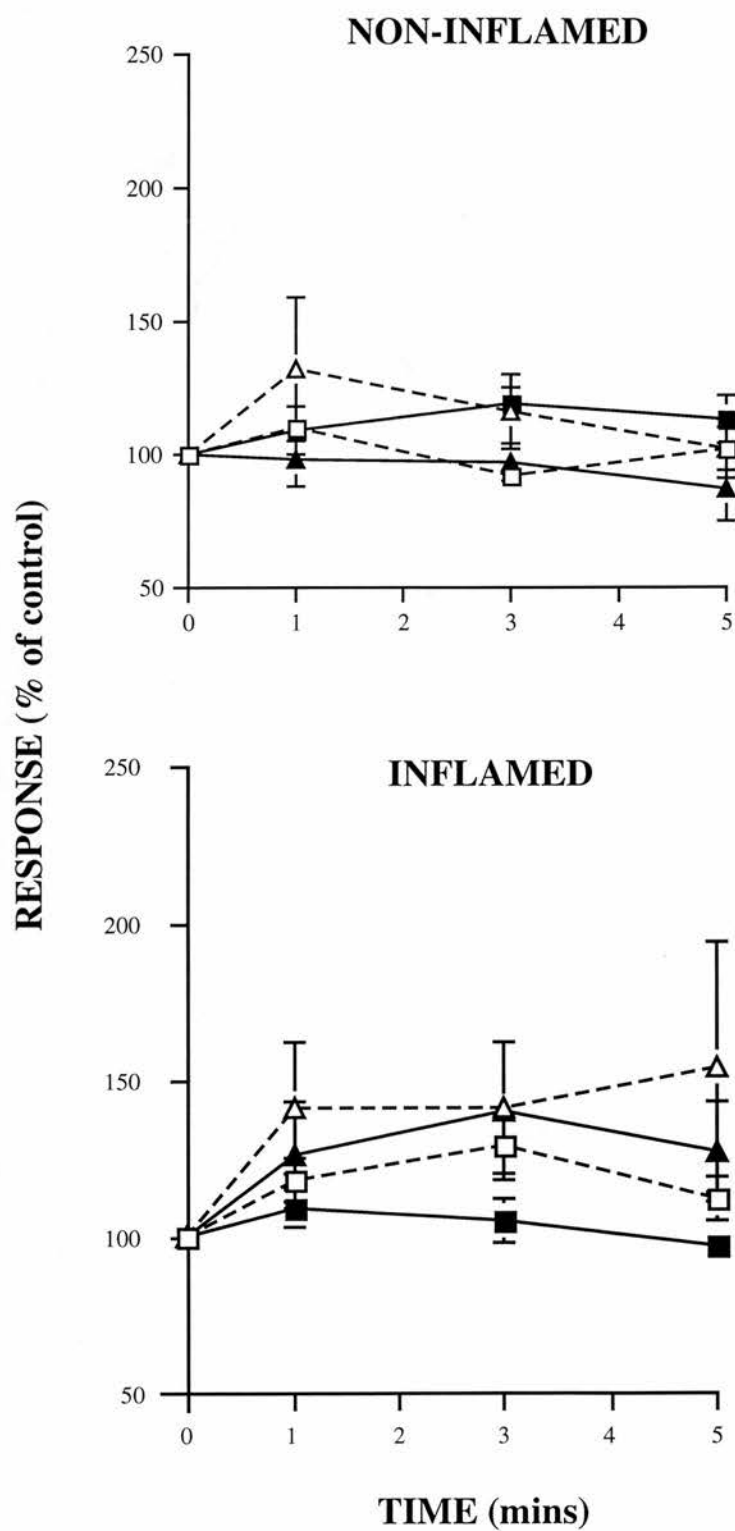
Mean thresholds from noxious mechanical and latencies from noxious thermal stimulation at time 0 in the non-inflamed group were  $9.5 \pm 0.9$  units<sup>#</sup> and  $4.4 \pm 0.5$  seconds respectively (n=18). In a separate group of animals with inflamed paws the pre-drug responses were  $2.7 \pm 0.2$  units and  $7.5 \pm 0.4$  seconds (carrageenan-induced reductions of  $82 \pm 18\%$  and  $37 \pm 2\%$ ; n=28).

GR82334, an NK<sub>1</sub> receptor antagonist, was without effect on paw withdrawal responses to both noxious mechanical and thermal stimulation of the paw. It failed to produce significantly different values from those of saline controls, in both the non-inflamed paw and the paw with a carrageenan-induced inflammation.

Statistical significance was evaluated by the Mann-Whitney U-test.

#units are described in section 5.2.4.1.

- Δ-- saline MECHANICAL threshold
- saline THERMAL latency
- ▲- GR82334, 3nmol MECHANICAL threshold
- GR82334, 3nmol THERMAL latency



**Figure 5.7.**

**The effect of intrathecal injection of L659,874 on paw withdrawal responses  
in the non-inflamed and inflamed paws**

Mean thresholds from noxious mechanical and latencies from noxious thermal stimulation at time 0 in the non-inflamed group were  $4.2 \pm 0.7$  units<sup>#</sup> and  $8.8 \pm 0.5$  seconds respectively (n=12). In a separate group of animals with inflamed paws the pre-drug responses were  $2.8 \pm 0.1$  units and  $6.8 \pm 0.1$  seconds (carrageenan-induced reductions of  $38 \pm 14\%$  and  $52 \pm 9\%$ ; n=28).

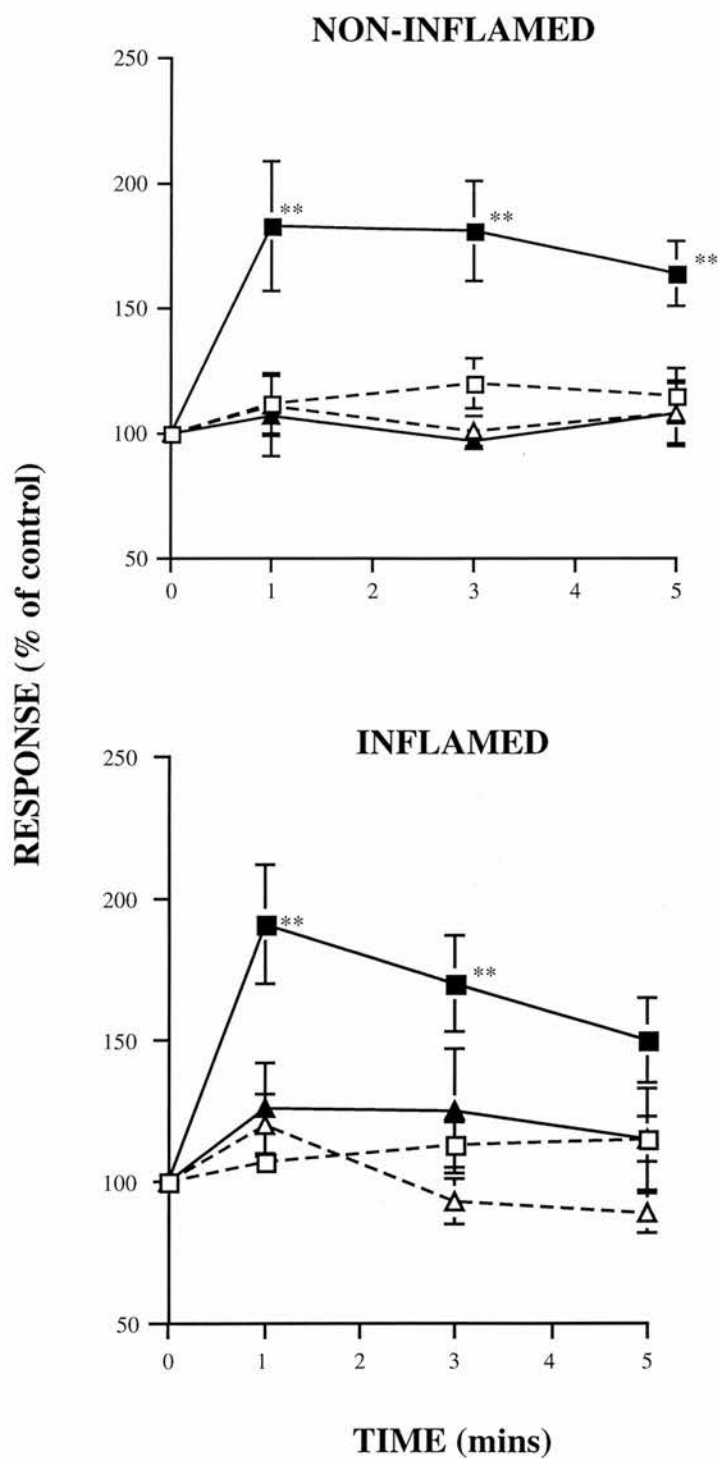
The NK<sub>2</sub> receptor antagonist L659,874 selectively increased withdrawal responses when the paw received a noxious thermal stimulus, but not noxious mechanical. The effect was significant, with respect to saline, in both the non-inflamed paw and the paw which received an injection of carrageenan 3 hours previously.

Statistical significance was determined using the Mann-Whitney U-test, and  $p < 0.01$ , with respect to saline, is denoted by \*\*.

#units are described in section 5.2.4.1.

- Δ-- saline MECHANICAL threshold
- saline THERMAL latency
- ▲-- L659,874, 3nmol MECHANICAL threshold
- L659,874, 3nmol THERMAL latency





**Figure 5.8.**

**The effect of intrathecal co-injection of GR82334 and L-AP3 on paw withdrawal responses in the non-inflamed and inflamed paws**

In the non-inflamed state the mean paw withdrawal thresholds to mechanical and latencies to noxious thermal stimulation were  $4.0 \pm 0.3$  units<sup>#</sup> and  $9.3 \pm 0.5$  seconds respectively (n=39). Whereas in a separate group of animals, carrageenan induced average decreases of  $73 \pm 34\%$  and  $48 \pm 9\%$  (mean pre-drug responses for this group being  $2.9 \pm 0.2$  and  $7.4 \pm 0.2$ ; n=58).

In the non-inflamed state the results of co-injection of GR82334 and L-AP3 were no different from the effect of either compound alone. However, when the metabotropic glutamate receptor and NK<sub>1</sub> receptor antagonists were co-administered in the inflamed state, the results were significantly different from the single injection of L-AP3: they were markedly reduced.

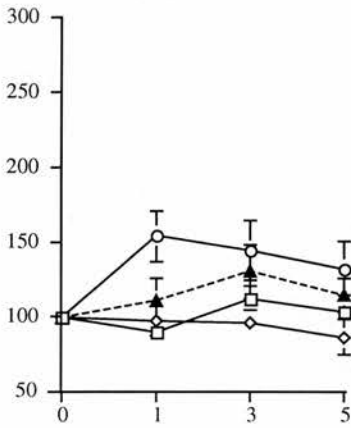
Significance was calculated using the Mann-Whitney U-test. \*\*=p<0.01 and \*=p<0.05, with respect to L-AP3 injected alone. Significance with respect to saline is not shown, see Figures 5.3.(a) and 5.6.

#units are described in section 5.2.4.1.

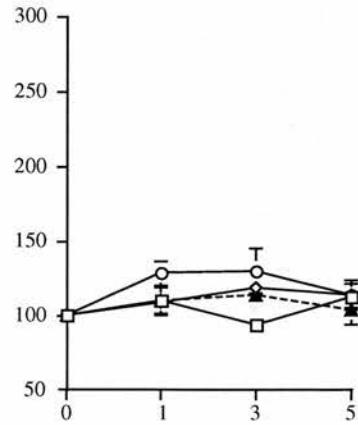
- saline
- ◇- GR82334, 3nmol
- L-AP3, 60nmol
- ▲- GR82334 + L-AP3

## NON-INFLAMED

### MECHANICAL

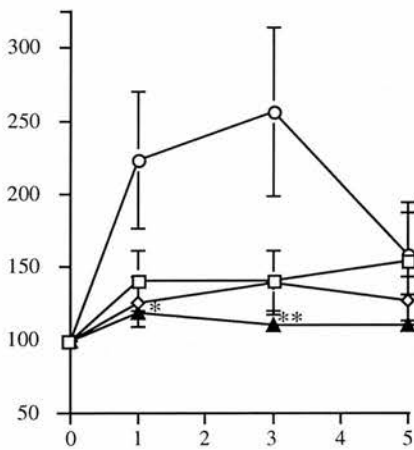


### THERMAL

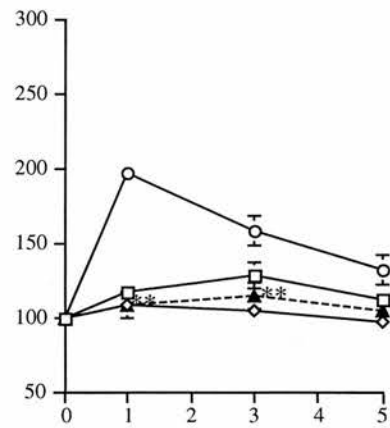


## INFLAMED

### MECHANICAL



### THERMAL



TIME (mins)

RESPONSE (% of control)

**Figure 5.9.**

**The effect of intrathecal co-injection of L659,874 and L-AP3 on paw withdrawal responses in the non-inflamed and inflamed paws**

In the non-inflamed state the mean paw withdrawal thresholds to mechanical and latencies to noxious thermal stimulation were  $4.0 \pm 0.3$  units<sup>#</sup> and  $9.4 \pm 0.6$  seconds respectively (n=32). Whereas in a separate group of animals, 3 hours of carrageenan-induced inflammation resulted in average decreases of  $56 \pm 26\%$  and  $56 \pm 12\%$  of withdrawal responses (mean pre-drug thresholds for this group being  $2.8 \pm 0.1$  units and  $6.6 \pm 0.2$  seconds; n=58).

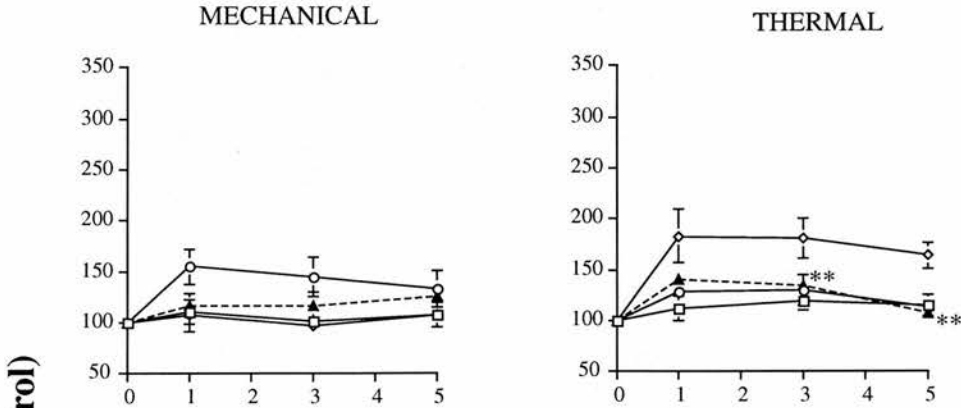
Co-injection of the metabotropic glutamate and NK<sub>2</sub> receptor antagonists made no difference in the response to noxious mechanical or thermal stimulation, with respect to the administration of single compounds.

Statistical analysis was carried out using the Mann-Whitney U-test. \*\*= $p < 0.01$  and \*= $p < 0.05$ , with respect to L-659,874 or L-AP3 alone. Significance with respect to saline is not shown, see Figures 5.3.(a) and 5.7.

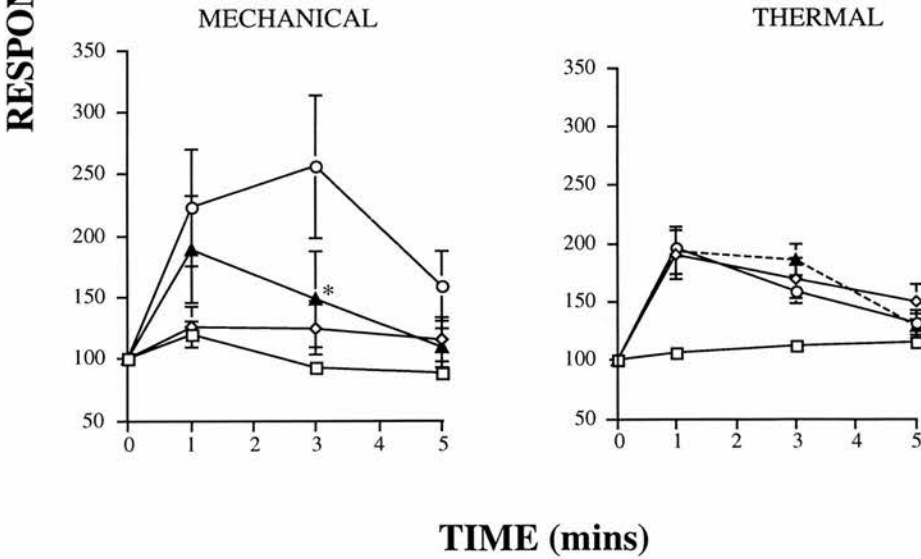
# units are described in section 5.2.4.1.

- DMSO
- ◇— L659,874, 3nmol
- L-AP3, 60nmol
- ▲— L659,874 + L-AP3

**NON-INFLAMED**



**INFLAMED**



**Figure 5.10**

**The effect of intrathecal co-injection of GR82334 and (S)-CHPG on paw withdrawal responses in the non-inflamed and inflamed paws**

In the non-inflamed state the mean paw withdrawal thresholds to mechanical and latencies to noxious thermal stimulation were  $4.2 \pm 0.2$  units<sup>#</sup> and  $9.5 \pm 0.4$  seconds respectively (n=40). Whereas in a separate group of animals, carrageenan-induced inflammation resulted in average decreases of  $64 \pm 34\%$  and  $48 \pm 10\%$  of responses (mean pre-drug thresholds for this group being  $2.7 \pm 0.1$  units and  $7.3 \pm 0.4$  seconds; n=57).

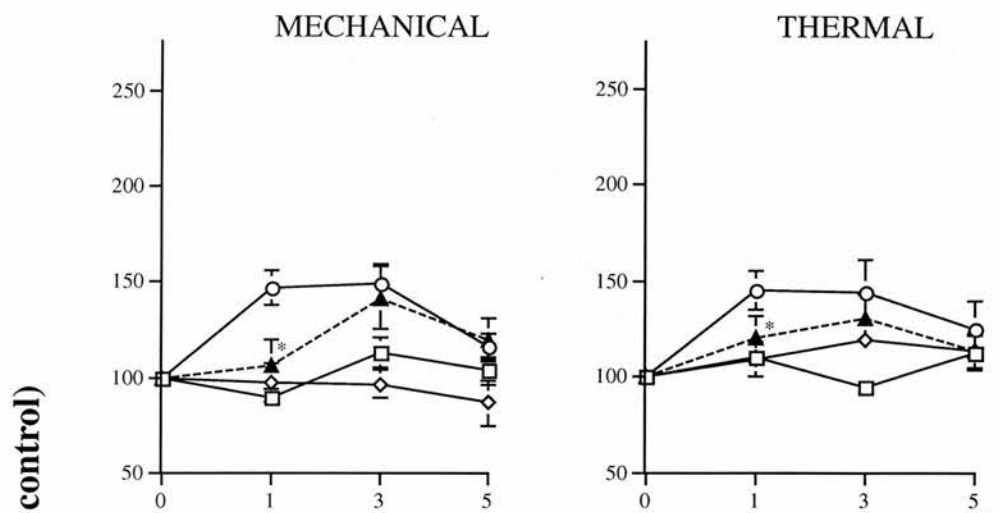
Similar to the results obtained when GR82334 was co-administered with the other metabotropic glutamate receptor antagonist L-AP3, the co-injection with (S)-CHPG led to a significant reduction in the effect seen with this compound alone - but in this case in the non-inflamed and inflamed paw.

Significance was calculated using the Mann-Whitney U-test. \*\*= $p < 0.01$  and \*= $p < 0.05$  with respect to (S)-CHPG injected alone. Significance with respect to saline is not shown, see Figures 5.5. and 5.6.

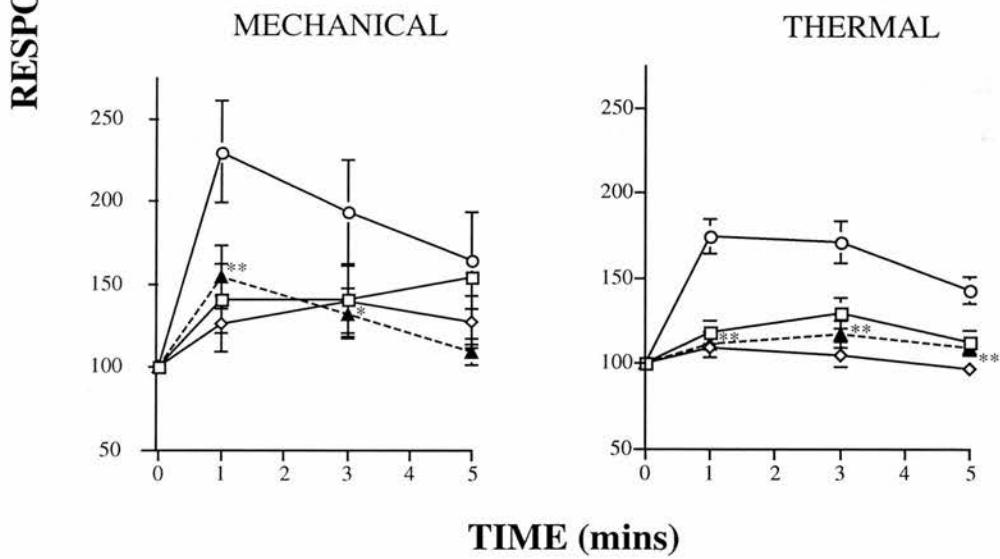
# units are described in section 5.2.4.1.

- saline
- ◇- GR82334, 3nmol
- (S)-CHPG, 48nmol
- ▲- GR82334 + (S)-CHPG

**NON-INFLAMED**



**INFLAMED**



**Figure 5.11.**

**The effect of intrathecal co-injection of L659,874 and (S)-CHPG on paw withdrawal responses in the non-inflamed and inflamed paws**

In the non-inflamed state the mean paw withdrawal thresholds to mechanical and latencies to noxious thermal stimulation were  $4.0 \pm 0.3$  units<sup>#</sup> and  $8.9 \pm 0.5$  seconds respectively (n=32). Whereas in a separate group of animals, carrageenan induced average decreases of  $46 \pm 16\%$  and  $42 \pm 10\%$  of withdrawal responses (mean pre-drug latencies being  $2.9 \pm 0.1$  units and  $7.2 \pm 0.4$  seconds; n=57).

Similar to the results obtained when L659, 874 was co-administered with the other metabotropic glutamate receptor antagonist, the co-injection with (S)-CHPG failed to alter the effects of each compound alone.

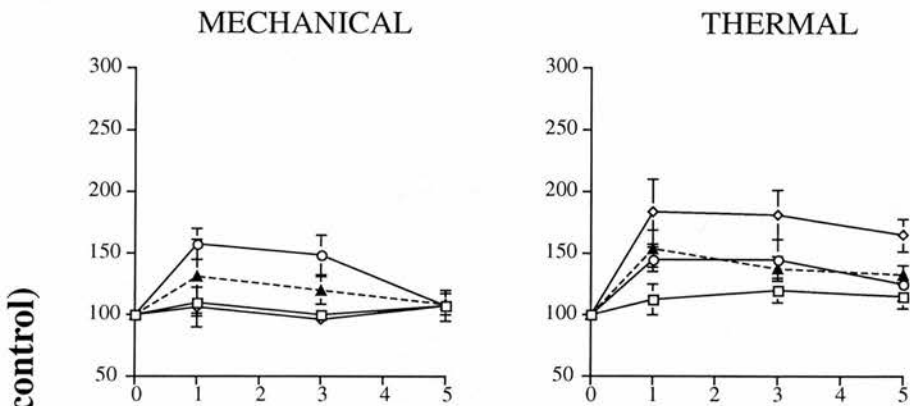
Statistical analysis was carried out using the Mann-Whitney U-test.  $**=p<0.01$  and  $*=p<0.05$  with respect to (S)-CHPG injected alone. Significance with respect to saline is not shown, see Figures 5.5. and 5.7.

<sup>#</sup> units are described in section 5.2.4.1.

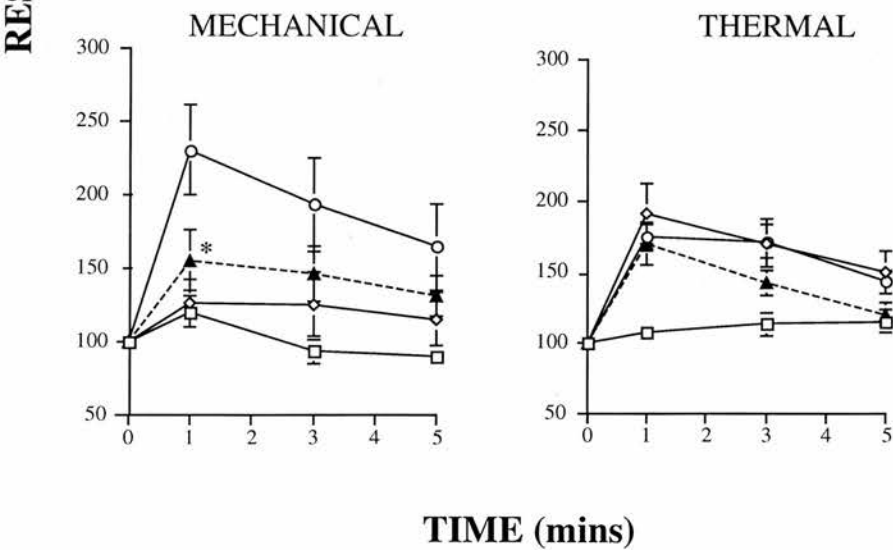
- DMSO
- ◇— L659,874, 3nmol
- (S)-CHPG, 48nmol
- ▲— L659,874 + (S)-CHPG



**NON-INFLAMED**



**INFLAMED**



## 5.4. DISCUSSION

The methods of behavioural testing employed in this study were based in the paw flick test originally described by Woolfe & MacDonald (1944), and the paw pressure test (Randall & Sellito, 1957). The method employed by Woolfe & MacDonald (1944) observed the glabrous surface of the foot, and was modified in the current study to test the hairy skin of the foot. This was easily accomplished by holding the animal in place with one hand (see Figure 5.2.). The dorsal hairy side of the foot was assessed because previous experiments (Chapter 3; Harris & Ryall, 1988; F.E. Munro PhD thesis, 1995) have highlighted the possibility that there may be a difference in sensitivity of the two skin types to certain noxious stimuli, e.g to mustard oil. This may relate to a sub-population of C afferent fibres in each skin type. Such testing was therefore used as a reliable method of monitoring drug-induced analgesia in the present investigation. Previous studies have shown, with the use of agonists, such as SP and glutamate, how the intrathecal method of drug delivery (Hylden & Wilcox, 1980) results in a dependable access of compounds to the spinal cord. Intrathecally administered NK and EAA receptor agonists give rise to compulsive biting and scratching of the caudal areas (Hayes & Tyers, 1979; Aanonsen & Wilcox 1986; 1987), i.e. those innervated by the spinal segments at which the injection is placed. These methods of behavioural testing and intrathecal drug administration were used in combination in the present experiments to assess the effect of mGlu, NK<sub>1</sub> and NK<sub>2</sub> receptor antagonists on the responses originating from hindpaws which received a noxious mechanical or noxious thermal stimulus. The study compared the results from naive animals with those which had received an intraplantar injection of carrageenan 3 hours earlier; a previously determined model of inflammation (Iadorola *et al*, 1988a; 1988b).

When saline, or 0.5% DMSO in saline (vehicle) were intrathecally applied a transient rise in latency/threshold to noxious thermal/mechanical stimuli was observed. Stress-induced analgesia may be evoked by a number of stimuli, both noxious and non-noxious (Bodnar *et al*, 1978; Hayes *et al*, 1978; Grau *et al*, 1981; Hamm *et al*, 1985), and there appear to be both opioid and non-opioid (including noradrenergic) components of this phenomenon (Chance & Schechter, 1979; Terman *et al*, 1984; Chance & Nelson, 1986). It is therefore feasible that the protocol in the current experiments was accompanied by a transient stress-induced endogenous analgesia. Due to this apparent procedure-evoked analgesia, all drug-induced alterations in responses were compared to the effects of vehicle, and not the pre-drug levels.

When mGluR antagonist (S)-CHPG was applied to animals with non-inflamed ('normal') paws there was a significant delay in the response to both noxious mechanical and thermal stimulation. However, the second antagonist used, L-AP3 (and its isomer D-AP3), had no such effect. Both of these antagonists act at Group I mGluRs, though (S)-CHPG is invariably more potent than L-AP3, and acts preferentially at mGluR1 (Pin & Duvoisin, 1995). This would suggest that the effects observed due to (S)-CHPG application were a result of the prevention of mGluR1 activation. Although L-AP3 was ineffective on responses from the non-inflamed paw, it was effective in inflamed paws (see below). Since the only cloned mGluR against which L-AP3 has been reported to be effective (but of low potency) is mGluR1 (Pin & Duvoisin, 1995) this is consistent with an involvement of mGluR1 in nociceptive inputs following carrageenan treatment.

Lambda-carrageenan has been used in previous studies to induce an acute state of inflammation, which peaks at around 3 - 4 hours and persists for several days (Iadorola *et al*, 1988a; 1988b). In the present study, animals received an intraplantar injection 3 hours prior to drug testing, following which the foot was observed to be red and swollen. Mechanical and thermal hyperalgesia in the sensitised animal were assessed by re-measuring withdrawal responses to noxious stimulation. Following the development of this inflammation, an intrathecal injection of both (S)-CHPG and L-AP3 resulted in a significant rise in withdrawal responses to both noxious mechanical and noxious thermal stimulation. The effect observed can be presumed to be mediated by mGluRs, as the stereoisomer of L-AP3, D-AP3, was still ineffective, compared to vehicle. Furthermore, the difference in responses between the two isomers was also statistically significant. Since (S)-CHPG was effective in both models assessed here, there appears to be a role for mGluR1 at least, in mediating brief noxious inputs under both normal and inflamed conditions. This is in agreement with the studies discussed earlier in Chapter 3, as both (S)-CHPG and L-AP3 attenuated the elevated activity evoked in dorsal horn neurons by topically administered mustard oil. On the basis of the present modestly-selective antagonists, it is not possible to exclude some additional contribution of mGluR5 and other mGluRs. Similarly, electrophysiological studies on thalamic nuclei have highlighted a nocispecific role for mGluRs, as antagonists did not affect responses induced by innocuous air jet stimuli, though they did reduce responses to noxious heating of the skin (Eaton *et al*, 1993a; 1993b). Furthermore, a study by Neugebauer *et al*, (1994b) provides congruent evidence in support of the inflammation-specific role for L-AP3 observed in the present study. In this electrophysiological investigation they found L-AP3 could attenuate responses elicited by pressure application to the knee, but only

following the induction of inflammation.

In contrast to the present work, no such role was found for L-AP3 in behavioural studies by Coderre's group (Coderre & Melzack, 1992; Coderre & Empel, 1994). Since the doses in the current experiments were based on the earlier study (Coderre & Melzack, 1992), dose differences are not the reason for the variances, although drug access could differ between the studies. The difference in timing is far more probable a cause, as Coderre & Melzack, (1992) tested animals some 15 minutes after the intrathecal injection. Clearly the current investigation provides evidence that the analgesic effect of L-AP3 (and (S)-CHPG) is very short-acting. This may be somewhat discouraging when considering the possibility of novel analgesic compounds. However, if a role for the receptors at which these drugs act can be elucidated, then identification of the target itself is a valuable tool for the further development of more longer-lasting analgesic compounds. In order to substantiate this evidence for mGluR involvement in nociception, further studies would be useful: electrophysiological experiments to assess the effect of mGluR antagonists on responses to brief noxious and non-noxious stimuli, preferably both before and after the application of mustard oil to define their role in specific sensitisation.

As well as glutamate, SP and NKA are found in some fine primary afferent fibres (Hökfelt, 1980; Ogawa *et al*, 1985). The role of both of these transmitters in nociception has been under much investigation over the past few decades. In the current investigation the effect of antagonists to NK<sub>1</sub> and NK<sub>2</sub> receptors (preferentially activated by SP and NKA respectively, (Regoli *et al*, 1987)) was assessed on brief noxious inputs, both before and after carrageenan-induced inflammation. When the NK<sub>2</sub> receptor antagonist L659,874 was administered intrathecally, there was a highly significant rise in withdrawal latency to the noxious thermal stimulus, though no such effect was observed on the threshold to pressure applied to the paw. This L659,874-mediated reflex depression was maintained for the duration of testing in the normal paw, and for the first two tests in the inflamed paw. These results are in complete agreement with previous electrophysiological experiments which have highlighted a selective role for NK<sub>2</sub> receptors in mediating brief noxious thermal inputs to thalamic and spinal cord dorsal horn neurons (Fleetwood-Walker *et al*, 1990; 1993; Santucci *et al*, 1993).

There have been very few published studies on the role of NK<sub>2</sub> receptors in behavioural testing. Lack of effect by intrathecally administered NK<sub>2</sub> receptor antagonist GR115211, on brief (tail flick and hot plate) and sustained (formalin) nociceptive inputs was observed by Birch *et al*, (1993). However, in that same study

GR115211 also failed to antagonise the effect of an intrathecally applied NK<sub>2</sub> agonist, indicating that the evidence does not reflect the genuine activity of NK<sub>2</sub> receptors. A more recent investigation, employing the non-peptide NK<sub>2</sub> receptor antagonist SR48968 found that NK<sub>2</sub> receptors appeared to be involved in formalin-evoked behaviours, though not brief nociceptive testing such as the tail flick test (Seguin *et al*, 1995). It is important to note however, that systemic administration of these compounds implies that they are not necessarily inducing their effect by acting at spinal cord receptors. Although this evidence is contrary to the current investigation, in that here a role for NK<sub>2</sub> receptors was observed in the brief paw-flick response, this is not entirely surprising as the tests utilised two different skin types. There is evidence that the glabrous side of the foot displays a somewhat different, lesser response to noxious stimulation, at least in the case of mustard oil, a selective C-fibre activator (Harris & Ryall, 1988; F.E. Munro PhD thesis, 1995). This may indicate that a different population of nociceptive afferent fibres innervate the two skin types, and that stimulus modality/intensity is a crucial factor in activating subpopulations of fibres.

The present investigation found no data to support a role for NK<sub>1</sub> receptors in the mediation of brief noxious inputs in either the normal animal, or carrageenan-treated animals. In further support of the data here, Schaible *et al*, (1990) demonstrated in an antibody microprobe study that SP could not be detected in the spinal cord of animals with a normal joint. Interestingly, even after a carrageenan/kaolin-induced inflammation, the average time that elapsed before SP was detected was 6.5 hours. In light of this evidence, it is hardly surprising that the current study found no effect of GR82334. It would appear that SP requires not only an inflamed state for release, but also quite some time under those inflammatory conditions. In addition to these studies, there are several electrophysiological experiments which are in agreement with the present data. Ionophoretic application of NK<sub>1</sub> antagonists do not appear to alter the firing evoked in dorsal horn neurons by either brief or medium-term noxious cutaneous stimulation (Fleetwood *et al*, 1987; 1990; Munro *et al*, 1993). Other electrophysiological studies have claimed that non-peptide NK<sub>1</sub> antagonist CP96,345 blocks spinal cord neuronal responses to "brief" noxious mechanical stimulation (De Koninck & Henry, 1991; Radhakrishnan & Henry, 1991). However, the stimulus used in these experiments was, arguably, far from short-lived and may have been subsequently tissue-damaging. A role for SP in nociceptive inputs following other forms of sensitisation, though not in normal preparations, is heavily supported by much electrophysiological data (Kellstein *et al*, 1990; Xu *et al*, 1992; Chapman & Dickenson, 1993; Laird *et al*, 1993; Thompson *et*



*al*, 1994). However, behavioural studies have yielded mixed results. There is a large body of evidence supporting the current data in that NK<sub>1</sub> receptors are not implicated in acute nociceptive tests (Garces *et al*, 1992; Malmberg & Yaksh, 1992a; Yamamoto & Yaksh, 1992a; Couture *et al*, 1993; Picard *et al*, 1993; Seguin *et al*, 1995). However, antagonists to NK<sub>1</sub> receptors are antinociceptive following some sustained noxious cutaneous inputs (Garret *et al*, 1991; Murray *et al*, 1991; Birch *et al*, 1992; 1993; Yashpal *et al*, 1993; Seguin *et al*, 1995), but not others (Yamamoto & Yaksh, 1992a). Undoubtedly the stimulus is a major factor in these contradictory reports, particularly if there is a possibility that each model is activating a different population of C- and A $\delta$ -afferent fibres (Treede *et al* 1992; see Fleetwood-Walker, 1995).

As there was no evidence for a role of SP in mediating brief noxious inputs in the present study, several considerations should be highlighted: firstly, there is evidence that the time required for SP release was inadequate following carrageenan administration (see Schaible *et al*, 1990). Secondly, recent single fibre-labelling studies have emphasised a lack of SP-LI in C polymodal fibres, in the guinea pig at least (Lawson *et al*, 1994). Since the existence of C polymodal afferent fibres in glabrous skin is dubious (see Treede *et al*, 1994), this may explain why other studies have previously detected a role for SP in the transmission on nociception from glabrous surfaces, i.e. the ventral paw and the tail. The current investigation has further drawn attention to the possibility that the innervation of these skin types may be crucially different, as the results obtained differ from the data available on glabrous skin.

Taken together, the results obtained using NK<sub>1</sub> and NK<sub>2</sub> receptor antagonists would suggest that, although SP and NKA are co-localised in the majority of instances (Dalsgaard *et al*, 1985) their roles upon release are really quite distinct. Studies of the effect of various stimuli on the detectable release of these peptides are highly supportive of such separate roles in sensory processing (Duggan *et al*, 1988; Duggan *et al*, 1990). NKA would appear to mediate a modality-specific nociception, in that it plays a role only in noxious thermal inputs to the spinal cord, under normal and sensitised conditions. On the other hand, SP seems to require a very much more prolonged input, or some form of sensitisation before it mediates nociceptive information. It is possible that upon the co-release of SP and NKA, initial events involve NKA in the process of inducing sensitisation in dorsal horn neurons, whereas SP then mediates the subsequent events which occur as a result of this phenomenon.

Some of the most fascinating results observed in this study were those elicited by the co-application of an mGluR antagonist and a NK antagonist. In short, the previously seen analgesic effects of the mGluR antagonists were occluded when either GR82334 or L659,874 were concurrently injected. Since the compounds were co-injected simultaneously, the results cannot be explained by re-setting of the baseline by one compound acting to pre-empt effects of the second. Furthermore, the phenomenon was seen both with compounds which were analgesic (NK<sub>2</sub>) and non-analgesic (NK<sub>1</sub>) in their own right. Previous electrophysiological intracellular recording studies investigating NMDA/NK receptor interactions have found the co-administration of agonists results in mixed effects (Randic *et al*, 1990; Rusin *et al*, 1992; 1993a; 1993b), though no such investigation has been done with antagonists. One study did however find that alone an NMDA or NK<sub>2</sub> antagonist partially abolished a capsaicin-evoked DRG response, but together they could almost completely attenuate it (Nagy *et al*, 1993). No such interactions with mGluRs and the tachykinins have yet been studied. The most likely explanation for the present data requires that a small amount of SP be released upon noxious stimulation. This SP is normally inadequate to mediate noxious inputs in the current model (perhaps due to rapid degradation) however it could be enough to act at other sites, for example a hypothetical inhibitory interneuron. Therefore, if the NK<sub>1</sub> receptor on this interneuron was blocked, the observed effect would be an increase in neuronal firing, which may be, at least partially, attenuated by the application of (S)-CHPG or L-AP3, thus manifesting as no change in the overall response. However, this would indicate that GR82334 alone should result in hyperalgesia, an effect not observed. A second possibility is that an intracellular interaction may be occurring. It is however difficult to envisage such an event that would lead to occlusion of analgesia, because both mGluR1/5 and NK<sub>1/2</sub> receptors work primarily through the same signal transduction cascades. It is possible that additional signals (which may be activated to different extents by the two receptor classes) are responsible. In general however, such explanations seem unlikely as the mGluR antagonists alone were analgesic, and why should cessation of NK<sub>1</sub> receptor activity negate this role? The case with NKA is somewhat different, as it is the L659,874-induced analgesia to noxious thermal stimuli, in the normal paw, which is reduced when either mGluR antagonists is co-administered. This may indicate that the input from the carrageenan-treated paw is too substantial to be altered. Interestingly though, L659,874 alone did not affect the withdrawal latency to noxious mechanical stimulation, but when co-injected with either mGluR antagonist in these carrageenan-treated animals, their previously analgesic effect was prevented. A further possibility is, of course that the drugs used

in this study interact themselves. If, for example, GR82334 is ineffective alone and then binds to, or interacts with L-AP3 or (S)-CHPG, then the overall observed response is equal to no injection at all. Further studies should be carried out, not only with antagonists, but also agonists, using a number of compounds with the greatest available specificity. These studies would aim to characterise (and if possible explain the mechanism of) the phenomenon and would ideally be carried out at behavioural, electrophysiological and biochemical levels. Whatever the mechanism, there appears to be a highly complex interaction occurring in the spinal cord when mGluR and NK receptors are simultaneously inhibited. These observations also underlie the point that problems can arise when using cocktails of drugs as analgesics, despite the fact that each is a wholly rational therapeutic stratagem in its own right.

In summary, it would appear that mGlu and NK<sub>2</sub> receptors are involved in the mediation of brief noxious inputs in both normal and carrageenan-treated animals. The role of NK<sub>1</sub> receptors in this model of inflammation appears to be linked to some kind of interaction with mGlu receptors, though the nature of this event is unknown.



## CHAPTER 6: SUMMARY AND CONCLUSIONS

Many previous studies have implicated both EAA and NK receptors in the mediation of sensory neurotransmission. The current investigation has, by means of electrophysiological recording experiments and, behavioural testing together with pilot experiments using a molecular biology approach; see Appendix 1, aimed to further elucidate the specific role of certain receptor subtypes activated by these primary afferent transmitters, and the cellular mechanisms by which they may act. The NMDA type of EAA receptor has been extensively studied, and there is a large body of evidence implicating it in the mediation of prolonged synaptic events, such as wind-up in the spinal cord (Davies & Lodge, 1987; Dickenson & Sullivan, 1987; 1990; Haley *et al*, 1990) and LTP in the hippocampus (see Collingridge, 1992). However, the role of mGluRs in sensory processing has not yet been adequately addressed. In the current study this was initially assessed by observing the effect of several mGluR antagonists on the responses of dorsal horn neurons evoked by sensory stimulation *in vivo*. Following this, several signal transduction pathways were investigated to reveal possible mechanisms through which mGluRs may operate. This study simultaneously addressed the role of the various second messengers in the neurotransmission of both innocuous and noxious information. In a further investigation, behavioural experiments were carried out to assess the effect of intrathecally applied mGlu, NK<sub>1</sub> and NK<sub>2</sub> receptor antagonists on withdrawal responses to noxious thermal and mechanical stimuli. Glutamate and SP are co-existing transmitters in fine primary afferent terminals (DeBiasi & Rustioni, 1988), suggesting that it is important to consider their effects in conjunction. Furthermore, evidence from previous electrophysiological experiments has highlighted the possibility of an interaction between EAA and NK receptors (Randic *et al*, 1990; Dougherty & Willis 1991b; 1993; Rusin *et al* 1992; 1993a; 1993b; Song & Zhao, 1994; Urban *et al*, 1994). Therefore, subsequent to an analysis of the effect of the individual compounds, the effect of co-injecting mGluR antagonists with each of NK<sub>1</sub> and NK<sub>2</sub> antagonists on the responses to noxious stimuli was monitored.

### (a) **Metabotropic Glutamate Receptors and Nociception**

The results obtained from the electrophysiological experiments in this thesis provide substantial evidence for a role of mGluRs in the mediation of noxious cutaneous inputs. Furthermore, it would appear that non-noxious inputs are not

mediated by this subtype of EAA receptors. The compounds used in the current study implicate group I mGluRs in the mediation of such nociceptive inputs to dorsal horn neurons. It would now be useful to observe the effects of the newer mGluR antagonists which have since become available (see Watkins & Collingridge, 1994; Pin & Duvoisin, 1995). Previous investigations have indicated a similar selective role for mGluRs in the transmission of noxious, but not non-noxious sensory inputs to thalamic nuclei (Eaton *et al*, 1993a; 1993b). The present data obtained from behavioural testing has highlighted that such a role for mGluRs in nociceptive processing is far more substantial when spinal cord neurons have been subjected to some form of sensitisation, in this instance a carrageenan-induced inflammation. These data are highly reflective of the role which the NMDA receptor plays in sensory neurotransmission, as NMDA receptors are similarly implicated in nociceptive processing in a sensitised state. Indeed, it is likely that these two receptor groups operate in concert to elicit the final output from dorsal horn neurons. Recent LTP investigations have hypothesised that mGluRs are involved in a conditional switch mechanism (Bortolotto & Collingridge, 1994), which could result in prolonged molecular events that gate the functional involvement of other receptors, for example the NMDA receptor.

The mechanisms which are instigated downstream of mGluR activation appear to be numerous. The present investigation found that at least three signal transduction targets, namely CamKII, PLA<sub>2</sub> and PKC, play specific roles in the mediation of high intensity inputs to the spinal cord. These pathways all demonstrated a clear link with group I/II mGluRs, as (1S,3R)-ACPD-evoked activity could be attenuated by their inhibitors, though the exact receptor subtype involved is not certain. CamKII and PKC have both been demonstrated to participate in LTP (Lovinger *et al*, 1987; Malinow *et al*, 1988; 1989; Malenka *et al*, 1989), a phenomenon with many analogies to the sensitisation which occurs in dorsal horn neurons, following the cutaneous application of mustard oil. It therefore seems likely that these signalling mediators will play comparable roles in the mediation of high intensity sensory inputs to the spinal cord, in this case via an mGluR. In addition to this, a role for PKC in nociception has been hypothesised on the basis of several experiments: this kinase has been shown to be involved in the modulation of NMDA receptor currents in the spinal cord and the hippocampus *in vitro* (Gerber *et al*, 1989; Ben-Ari *et al*, 1992). Ionophoresis of the same PKC inhibitors utilised in the current investigation markedly reduced the sustained activity in dorsal horn neurons elicited by the cutaneous application of mustard oil (Munro *et al*, 1994a). Also, [<sup>3</sup>H]PDBu binding assays have demonstrated how sustained noxious stimulation results in the

translocation of PKC to the membrane compartment, in the spinal cord (Mao *et al*, 1992; 1993; Munro *et al*, 1994a; Yashpal *et al*, 1995). Thus the present investigation supports the hypothesis that PKC is a crucial mediator of the cellular signalling involved subsequent to a nociceptive input to the dorsal horn. Several other studies have highlighted the possibility that PLA<sub>2</sub> and consequently formed eicosanoids may also be an important mediator of nociception in the central nervous system, as well as in the periphery (Chapman & Dickenson, 1992; Malmberg & Yaksh, 1992a; 1992b; Coderre & Yashpal, 1994; Boyce *et al*, 1994). The present study supports such a central role for this signalling mediator, particularly in the mediation of sustained noxious inputs. The work presented here could not provide any conclusive evidence as to the nature of the role which PKA or non-receptor tyrosine kinases may play in sensory inputs to the spinal cord.

#### **(b) NK<sub>2</sub> Receptors and Nociception**

Previous electrophysiological studies have demonstrated the specific mediation of noxious thermal, but not mechanical inputs to thalamic and spinal neurons by NK<sub>2</sub> receptors (Fleetwood-Walker *et al*, 1990; 1993; Santucci *et al*, 1993). Since the NK<sub>2</sub> receptor antagonist L659,874 was analgesic in the thermal paw flick, but not paw pressure test, the current work similarly concludes that NK<sub>2</sub> receptors, and thus probably NKA, are involved in a modality-specific mediation of both brief and sustained thermal and chemical, but not mechanical noxious inputs to the spinal cord.

#### **(c) NK<sub>1</sub> Receptors and Nociception**

The present investigation found no behavioural evidence for a role for NK<sub>1</sub> receptors in mediating the inflammatory inputs induced by intraplantar carrageenan. This is somewhat surprising given that there is a large body of literature supporting a role for SP in mediating chronic noxious input (Kellstein *et al*, 1990; Birch *et al*, 1992; 1993; Xu *et al*, 1992; Chapman & Dickenson, 1993; Laird *et al*, 1993; Yashpal *et al*, 1993; Thompson *et al*, 1994). There is a possibility that the hairy skin type which was tested may be innervated by a distinct population of fibres which do not contain SP. This is unlikely however, as NK<sub>2</sub> antagonist L659,874 was observed to be analgesic and NKA is highly localised with SP in DRG cells (Dalsgaard *et al*, 1985). Therefore, if SP is indeed being released it would appear to be either metabolised or degraded too quickly to exert an obvious effect in the current investigation. Previous antibody microprobe investigations support this conclusion, as SP is not detected for an average of 6.5 hours after a carrageenan/kaolin-induced

inflammation (Schaible *et al*, 1990) and a study which utilised peptidase inhibitors demonstrated how SP could be detected more quickly and widespread if its degradation was prevented (Duggan *et al*, 1992). The current model could of course be inappropriate to invoke a substantial amount of NK<sub>1</sub> receptor involvement. Results similar to the present experiments were obtained in an electrophysiological study in which NK<sub>2</sub>, but not NK<sub>1</sub> receptor antagonists could inhibit the sustained activation of dorsal horn neurons elicited by mustard oil (Munro *et al*, 1993), therefore several stimuli are apparently insufficient to observe the involvement of SP in nociception.

**(d) Metabotropic Glutamate and NK Receptor Interactions in Nociception**

Although previous electrophysiological studies have hypothesised an interaction between EAA and NK receptors (Randic *et al*, 1990; Rusin *et al*, 1992; 1993a; 1993b; Urban *et al*, 1994), none of them have had sufficient data to elucidate the mechanism by which such an interaction may be occurring. The current behavioural study also suggests that there is some form of interaction taking place, (though it is impossible to ascertain the mechanism without further experiments) since concurrent blockade of mGlu and NK receptors leads to a loss of mGluR-mediated analgesia.

The work presented here has highlighted many features of nociception. Metabotropic glutamate receptors, particularly mGluR1/5, appear likely to mediate (at least in part) A $\delta$ - and C-fibre inputs to the spinal cord, especially in a sensitised state. Furthermore, this may be accomplished through several signal transduction mechanisms, likely to include PKC, CamKII and PLA<sub>2</sub> as downstream mediators. Neurokinin<sub>2</sub> receptors are apparently involved in brief and sustained noxious thermal but not mechanical inputs, whereas NK<sub>1</sub> receptors appear to require a very substantial, sustained noxious input before their role is appreciated.

The current study does highlight the possibility of an interaction between EAA and NK receptors, but the complex nature of this means the likelihood of drug combinations as efficient analgesics still requires many years of study.

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## APPENDIX 1:

### INVESTIGATION OF THE EFFECT OF METABOTROPIC GLUTAMATE RECEPTOR ANTAGONISTS ON COMPLETE FREUND'S ADJUVANT-INDUCED C-FOS mRNA EXPRESSION IN THE SPINAL CORD

#### A.1. AIMS

Following the induction of inflammation due to a subcutaneous injection of complete Freund's adjuvant (CFA), c-fos expression is known to increase in the spinal cord. In order to assess the role of mGluRs in this increased gene expression, antagonists were applied intrathecally during the first four hours of inflammation. The effect of CFA on c-fos mRNA expression and ultimately any changes induced by mGluR antagonists were monitored by slot blot hybridisation to poly(A)<sup>+</sup> extracts from whole spinal cord.

#### A.2. METHODS

##### A.2.1. Tissue Preparation

Adult male Wistar rats (320-770g) were anaesthetised and cannulated as in section 3.2.2. An incision was made into the dorsal surface of the neck, over C2/C3 and a minor laminectomy was performed in order to expose C2 - C3. After the dura was carefully cut a, polyethylene cannula (2 French Gauge: outer diameter of 0.70mm, inner diameter of 0.38mm, with green luer fitting; Portex, Hythe, UK) was filled with saline or drug and inserted into the intrathecal space between C2 and C3. It was then eased along the spinal cord until the caudal tip reached the region of L2 - L3. This position was determined by a mark made on the cannula prior to its insertion, and usually confirmed by reflex twitching of the hindlimbs. At this point (designated as time 0), two injections were given simultaneously. Firstly, a 150µl subcutaneous injection of complete Freund's adjuvant (CFA; 1.0mg/ml killed *Mycobacterium tuberculosis* suspended in 85% paraffin oil / 15% mannide monooleate; Sigma Chemical Company, Poole, UK) was given to both feet. This particular CFA was used since it produces an inflammation very quickly and is known to induce c-fos expression in the spinal cord after only 4 hours (Lantéri-Minet *et al*, 1993b). The peripheral response to CFA was monitored in part by measuring the circumference of the animal's paw at 0,1 and 4 hours. Secondly, 50µl of saline or drug were given via the cannula into the intrathecal space. Inflammation was

allowed to develop for a further 4 hours, with the intrathecal injection repeated at hourly intervals. During this time anaesthesia was maintained with  $\alpha$ -chloralose using the same criteria described in section 3.2.2. After 4 hours a laminectomy at L1-L5 was carried out and two pieces of spinal cord (L1-L2 midway and L2 midway-L5) were removed bilaterally, each weighing approximately 100mg. They were immediately frozen in liquid nitrogen, where they were stored until required for RNA extraction.

#### **A.2.2. Drugs**

Two metabotropic glutamate receptor antagonists were given intrathecally: L-AP3 and (S)-CHPG, 30mM in aqueous solution, pH7.0.

#### **A.2.3. RNA Extraction**

The extraction of RNA was carried out using a QuickPrep® *Micro* mRNA purification kit (Pharmacia Biotech, St Albans, UK). All procedures were carried out under RNase free conditions. This entailed either autoclaving or washing equipment in absolute alcohol followed by several washes in diethyl pyrocarbonate (DEPC)-treated water. All solutions were at room temperature, unless otherwise stated. The tissue was removed from liquid nitrogen and immediately homogenised in a buffered aqueous solution containing guanidinium thiocyanate (GTC) and N-lauroyl sarcosine. The extract was then diluted three-fold with 10mM Tris-HCl (pH 7.5)/ 1mM EDTA and homogenised further. The sample was centrifuged (13,000g, 3 minutes) and the supernatant added to oligo(dT)-cellulose in a microcentrifuge tube. The tube was manually inverted for 3 minutes to allow poly (A)<sup>+</sup>RNA to bind to the oligo(dT)-cellulose. Following a 10 second centrifugation this supernatant was removed and 1ml of a high salt buffer (10mM Tris-HCl (pH 7.5)/ 1mM EDTA/ 0.5M NaCl) was added. Five sequential batch washes with this buffer and then two with a low salt buffer (10mM Tris-HCl (pH 7.5)/ 1mM EDTA/ 0.1M NaCl) took place. Each wash was accomplished by a process of resuspension and 10 second recentrifugation. After the last low salt wash, the pellet was resuspended in 0.3ml of low salt buffer then transferred to a MicroSpin Column and washed a further 3 times with 0.5ml low salt buffer. Finally, the polyadenylated material was eluted with two applications of 0.2ml of 10mM Tris-HCl/1mM EDTA which had been warmed to 65°C.

#### **A.2.4. Quantitation and Precipitation of RNA**

Concentration and purity of the RNA samples was evaluated by comparing spectrophotometer readings at 260nm and 280nm. Samples were placed in RNase free quartz cuvettes and readings were taken from a Shimadzu UV-1201 spectrophotometer (VA Howe & Co. Ltd., Banbury, UK). Before storing the RNA at -70°C it was necessary to precipitate it. This was done by adding 10µg of glycogen in DEPC-treated water and 40µl of 2.5M potassium acetate, then storing under ethanol at -20°C for a minimum of 30 minutes. Finally after 5 minutes of centrifugation (13,000g, 4°C) the samples were stored under ethanol at -70°C until needed.

#### **A.2.5. Slot Blot Assay**

##### **A.2.5.1 3' Labelling of the Oligonucleotide Probe**

*c-fos antisense 48 base oligomer:* 5' GAG CGT ATC TGT CAG CTC CCT CCT CCG ATT CCG GCA CTT GGC TGC AGC. This *c-fos* oligomer is complementary to the base sequence 582-630 of rat *c-fos* mRNA (Curran *et al*, 1987) which corresponds to the conserved DNA binding site in Fos proteins. This means that proteins closely related to *c-fos* could also be detected. For the  $\beta$ -actin controls (see section A.2.7.) the following sequence, complimentary to amino acids 135 - 150 of rat  $\beta$ -actin mRNA, was used: 5' GCC AGT GGT ACG ACC AGA GGC ATA CAG GGA CAA CAC AGC CTG GAT CCG (Nudel *et al*, 1983; see Figure A.2).

The following reaction mixture was warmed in a sterile eppendorf tube at 32°C for 15 minutes: 3µl of DEPC treated water containing 0.68µg (38pmol) of antisense *c-fos* probe, 5µl of terminal deoxynucleotidyl transferase (TdT, 15 units/µl), 10 µl potassium cacodylate tailing buffer, 5µl [<sup>35</sup>S]dATP $\alpha$ S (70pmol; 1-1.3 x 10<sup>3</sup> Ci/mmol) and 38µl of DEPC treated water to make a final volume of 61µl. This volume was calculated to ensure the TdT was diluted at least 1 in 10. The mixture was briefly vortexed for before being spun down (13,000g, 2-3secs) and then incubated with gentle

**Figure A.1.**

**Nucleotide sequence of rat *c-fos***

The nucleotide sequence reads in the 5' to 3' direction (from Curran *et al*, 1987). The oligomer probe used was complimentary to the base sequences 582-629, as shown in italics above the open reading frame.

CGCAACTGAGAAGACTGGATAGAGCCGGCGGAGCCGCGAACGAGCAGTGACCGCGCTCCCACCCAGCTCTGCTCTGCA  
GCTCCCACCAAGTGTCTACCCCTGGACCCCTCGCCGAGCTTTGCCCAAACCACGACCATGATGTTCTGCGGTTTCAACGC  
GGACTACGAGGCGTCATCCTCCCGCTGCAGTAGCGCTCCCCGGCCGGGGACAGCCTTCTCTACTACCATTCCCCAGCC  
GACTCCTTCTCCAGCATGGGCTCCCTGTCAACACACAGGACTTTTGCGCAGATCTGTCCGTCTCTAGTGCCAACCTTAT  
CCCCACGGTGACAGCCATCTCCACCAGCCCAGACCTGCAGTGGCTGGTGCAGCCCACTCTGGTCTCCTCCGTGGCCCCA  
TCGAGACCAGAGCGCCCCATCCTTACGGACTCCCCACCCCGTCGACCGGGGCTTACGCCAGAGCGGGAGTGGTGAAG  
ACCATGTCAGGCGGCAGAGCGCAGAGCATCGGCAGAAGGGGCAAAGTAGAGCAGCTATCTCCTGAAGAGGAAGAGAA

**CGACGTCGGTTCACGGCCTTAGCCTCCTCCCTCGACTGTCTA**

ACGGAGAATCCGAAGGGAAGGAAT<sup>580</sup>AAGATGGCTGCAGCCAAGTGCCGGAATCGGAGGAGGGAGCTGACAGT  
**TGCGAG**

**ACGCTC**CAAGCGGAGACAGATCAACTTGAAGACGAGAAG<sup>640</sup>TCTGCGTTGCAGACCGAGATTGCCAATCTACTGAAA  
GAGAAGGAAAAAAGTGGAGTTTATTTTGGCAGCCCACCGACCTGCCTGCAAGATCCCCAATGACCTGGGCTTCCCAGAGG  
AGATGTCTGTGACCTCCCTGGACTTGACTGGGGGTCTGCCTGAGGCTACCACCCCAGAGTCTGAGGAGGCCTTACCCT  
GCCTCTTCTCAATGACCCTGAGCCCAAGCCATCCTTGGAGCCGGTCAAGAACATTAGCAACATGGAGCTGAAGGTGAA  
CCCTTTGATGACTTCTTGTTCGGCATCATCTAGGCCAGTGGCTCGGAGACTGCCCCTCTGTGCCAGATGTGAGACC  
TGTCTGGTTTCTTCTATGCAGCAGACTGGGAGCCTCTGCACAGCAGTTCCTTGGGGATGGGGCCCATGGTCACAGAGCT  
GGAGCCCCCTGTGACTCCCGTGGCACCTGCACTCCAGCTGCACTACCTATACGTCTTCTTTGTCTTACCTACCCCGA  
GGGTGACTCCTTCCCTAGCTGCGCAGCTGCCACCGAAAGGGCAGCAGCAGCAACGAGCCCTCCTCTGACTCAGTGAG  
CTCGCCACACTGCTAGCCCTGTGAGCAGTCAGAGAAGGCAGGGCAGCCGGCACTGACTGAGCTGGTGCATTAGAGAG  
AGGAGAAACACGTCTTCCCTGCAGGGGTTCCTGTAGACCTAGGGAGGACCTTATCTGTAGTGAACACACCAAGCTGTG  
GACCTCAAGGACTTGAAAGCATCCACATCTGGACTCCAGTCCTACCTCTTCCGCAGATGTAGCAAAAAACAAAAA  
ACAAAACAAAAAACAAAAAATCAAAAGCAACCGCATGGAGTGTATGGTTTGTAGTGACACCTGAGA  
GCTGGTAGTTAGTAGCATGTGAGCCAGGCTGGGTCTGTGTCTCTTTCTCTTCTCCTTAGTCTTCTCATAGCATTAACT  
AATCTGTTGGGTTCAATTATTGGAATTAACCTGGTGTGGATATTTTTCGGATTGTATCTAGTGCAGCTGATTTTAACAATA  
CCTACTGTGTTTCTGGCAATAGTGTGTCCAATTTAGAAATGACCAATATTAACTAAGAAAAGATAGAAGCTTATTTTCC  
GGTAGATAGAAATAAATCGCTATATCCACGTACTGTAGCTCTTACGCGTCCATGTTTATTGTACATGTAAGTATCATGC  
ATTGTTGAGGTGGTCTGAATGTTCTGACATTAACAGTTTTCCATGAAAACGTTTTATTGTGTTTTCAATTTATTTATTAAG  
ATGGATTCTCAGATATTTATATTTTATTTTATTTTCTATCCTGAGGTCTTTCGACATGTGGAAAGTGAATTTGAATGA  
AAAAATTTTAAGCATTGTTTGCTTATTGTTCCAAGACATTGTCAATAAAAGCATTTAAGTTGAATGCGAAAAA  
AAAAA

**Figure A.2.**

**Nucleotide sequence of rat  $\beta$ -actin**

The nucleotide sequence reads in the 5' to 3' direction (from Nudel, *et al*, 1983). The oligomer probe used was complimentary to amino acids 135-150, as shown in italics above the open reading frame.

5'GCTGCGCTCGTCGTCGACAACGGGTCCGCCATGTGCAAGGCCGGCTTCGCGGGCGACGATGCTCCCCGGGCCGTCTT  
CCCCTCCATCGTGGGCCGCGCCTAGGCACCAGGGTGTGATGGTGGGTATGGGTCAGAAGGACTCCTACGTGGGCGACG  
AGGCCCAGAGCAAGAGAGGCATCCTGACCCTGAAGTACCCCATGAACACGGCATTGTAACCAACTGGGACGATATGG  
AGAAGATTTGGCACCACACTTTCTACAATGAGCTGCGTGTGGCCCCTGAGGAG<sup>100</sup>CACCCTGTGCTGCTCACCGAGGCC  
CCTCTGAACCCTAAGGCCAACCCTGAAAAGATGACCCAGATCATGTTTGAGACCTTCAACACCCAGCCATGTACGTA

**CGGTAGGTCCGACACAACAGGGACATACGGAGACCAGCATGGTGACCG**

**GCCATCCAGCGTGTGTTGTCCCTGTATGCCTCTGGTTCGTACCACTGGCATTGTGATGGACTCCGGAGACGGGGTC**  
ACCCACACTGTGCCCATCTATGAGGGTTACGCGCTCCCTCATGCCATCCTGCGTCTGGACCTGGCTGGCCGGGACCTGA  
CAGACTACCTCATGAAGATCCTGACCGAGCGTGGCTACAGCTTC<sup>200</sup>ACCACCACAGCTGAGAGGGAAATCGTGCGTGA  
CATTAAAGAGAAGCTGTGCTATGTTGCCCTAGACTTCGAGCAAGAGATGGCCACTGCCGCATCCTCTTCCTCCCTGGAG  
AAGAGCTATGAGCTGCCTGACGGTCAGGTCATCACTATCGGCAATGAGCGGTTCCGATGCCCCGAGGCTCTCTTCAGC  
CTTCCTTCCTGGGTATGGAATCCTGTGGCATCCATGAACTACATTCAATTCCATCATGAAGTGTGACGTTGACATCCGT  
AAAGACCTCTATGCCAACACAGTGCTGTCTGGTGGC<sup>300</sup>ACCACCCTGTACCCAGGCATCGCTGACAGGATGCAGAAGG  
AGATTACTGCCCTGGCTCCTAGCACCATGAAGATCAAGATCATTGCTCCTCCTGAGCGCAAGTACTCTGTGTGGATTGGT  
GGCTCTATCCTGGCCTCACTGTCCACCTTCCAGCAGATTTGGATCAGCAAGCAGGAGTACGATGAGTCCGGCCCCCTCCA  
TCGTGCACCGCAAATGCTTCTAG 3'



agitation for 1 hour at 32°C. To optimise conditions, a further 5µl of <sup>35</sup>S-labelled ATP and 2µl of TdT were added at the end of the first hour, this combination was then allowed another hour's incubation period. The reaction was stopped by cooling on ice for 15 minutes.

At this time two 1µl samples were removed from the tube. These were compared with two later samples taken after the remainder of the mixture had been purified from unincorporated nucleotide using a Nu-Clean D25 disposable spun column (International Biotechnologies Inc, New Haven, USA). This was done by placing the sample onto the surface of the gel in the column and then centrifugation for 4 minutes at 1,100g, with collection into a fresh tube. 1ml of scintillation fluid was added to the 1µl test samples in sterile eppendorf tubes and counts per minute were read using a Wallac 1450 Microbeta Plus liquid scintillation counter with customised software (Department of Veterinary Pathology, University of Edinburgh). The difference between the pre-spun and post-spun samples allowed the percentage incorporation of the radiolabel to be estimated. The labelled probe was stored at -70°C until required, for a maximum of 14 days.

#### **A.2.5.2. Denaturation and Fixation**

A 48 well Bio-dot Slot Format Microfiltration unit (Biorad Laboratories, Inc., Hemel Hempstead, UK) was used in which each well was 7mm by 0.75mm. Before using the apparatus it was washed once in 95% alcohol followed by two rinses in DEPC water. The apparatus was assembled according to the manufacturer's recommendations, with a Zeta-Probe nylon membrane (9cm by 12cm) which had been pre-wetted in DEPC-treated water. The outer wells were not used, to avoid any potential loss of sample through uneven vacuum effects. These wells had buffer applied when the others had samples placed in them. Since the samples contained such variable levels of RNA, each was adjusted to contain the amount known to be in the least concentrated sample. This was accomplished by diluting the stronger samples with 1mM EDTA. Such uniformity was necessary for true comparison at the grey scale analysis stage.

Following their removal from the freezer, samples were re-centrifuged (5 minutes, 13,000g) and the ethanol was removed using a sterile glass pipette. They were then redissolved in 0.5ml of ice cold 10mM NaOH/1mM EDTA, and diluted where necessary (see above). To prepare the membrane, 0.5ml of DEPC water was placed in each well, then a vacuum applied. Next the 0.5ml samples themselves were added to the

wells and a vacuum applied until drying could be seen. The samples were washed through with 0.5ml of buffer and a final vacuum. Upon removal the membrane was immediately washed in 2 x standard sodium citrate (1 x SSC = 150mM NaCl, 15mM trisodium citrate)/ 0.1% sodium dodecyl sulphate (SDS) and blotted lightly.

#### **A.2.5.3. Hybridisation**

The membrane was carefully placed in a sterile plastic container with 200ml of a prehybridisation buffer consisting of 10x Denhardt's Solution, (1 x Denhardt's Solution = 0.02% each of bovine serum albumin, polyvinylpyrrolidone and Ficoll) 5 x SSC, 20mM NaH<sub>2</sub>PO<sub>4</sub>, 7% SDS and 100µg/ml denatured salmon sperm DNA (all final concentrations) and left in a shaking water bath at 68°C overnight (a temperature derived from standard calculations which consider both the base-pair content and the type of probe used). The following day the buffer was removed and replaced with a fresh, but identical, solution. 55-60µl of the 3' end <sup>35</sup>S-labelled oligonucleotide probe complementary to c-fos was added (4.5 - 5.8 x 10<sup>5</sup> c.p.m./ml buffer) and allowed to hybridise with the membrane, 68°C, overnight. The membrane was then washed twice in 3 x SSC, 10 x Denhardt's, 5% SDS and 25mM NaH<sub>2</sub>PO<sub>4</sub> and once in 1 x SSC, 1% SDS, (30 minutes each wash, 50°C).

#### **A.2.6. Autoradiography**

The blot was allowed to dry naturally (2 - 3 hours, room temperature) before being apposed to X-ray film (Kodak XAR-5, Biorad Laboratories, Inc., Hemel Hempstead, UK), with two intensifying screens, at -70°C for 12 - 14 days. It was developed using a standard X-ray developing machine (Small Animal Clinic, Summerhall, Edinburgh).

#### **A.2.7. Controls**

In order to determine the levels of c-fos mRNA expressed under various conditions, several controls were necessary for these experiments. Firstly, a surgery/vehicle control whereby the animal underwent an intrathecal cannula insertion and received hourly injections of saline. Secondly, for direct comparisons with drug-effects, further animals received the same treatment as above along with a bilateral injection of CFA into their feet. A third group had only intravenous and tracheal cannulae inserted, then were left for 4 hours. This was to determine levels of c-fos under

the influence of minimal noxious inputs. Finally, to observe the effect of the CFA alone, one animal was given the bilateral injections into the feet, but did not undergo back surgery.

In order to compare one slot with another, controls for levels of mRNA bound to the membrane were also carried out. This entailed stripping the membrane immediately after the exposure which determined levels of c-fos mRNA expression. This was accomplished by washing it 2 times, for 30 minutes each in a 0.1 x SSC/0.5% SDS solution at 95°C, followed by overnight exposure at -70°C. The membrane was subsequently re-assayed and treated in the same manner as before (section A.2.5.), but in this case was probed for  $\beta$ -actin, a standard cellular gene which should have uniform expression, regardless of treatment (Iadorola *et al*, 1988a). Should the levels of  $\beta$ -actin expression differ, they should be taken into account when analysing the levels of c-fos, i.e. if one slot contains twice as much mRNA than the next then the levels of c-fos expression should be halved for comparison.

#### **A.2.8. Analysis of Results**

Initial attempts were made to analyse the autoradiographs by means of an image analysis programme, customised to detect grey levels from silver grain autoradiographs. Using Image 1.44 VDM-F (in an Apple Macintosh M1298) a 'live' image from a camera above the autoradiograph allowed each slot to be captured as an image. A region of interest (ROI), usually encompassing the entire slot, was analysed for grey levels. An identical ROI immediately adjacent to the slot was then measured in the same manner for background levels. This method proved unsuitable for the measurements required, as it relied too much on human judgement of the slot position. Therefore, a further program was used, Image 1.49, which utilised a scanned and magnified image of the autoradiograph and counted pixels per ROI. An ROI was set and used for each slot in turn, and the background adjacent to each slot was likewise measured. To minimise the margin of error a mean of all 23 background measurements was used to compare with the levels from each slot.

Unfortunately although the  $\beta$ -actin mRNA controls could be analysed in this manner the levels of c-fos which were expressed throughout the blot in these pilot studies were too low to evaluate quantitatively, even with a magnified scanned image. As the slots were discernible by eye, it was decided that each slot would be given an empirical rank from 0 to 3 in order to determine any trends in c-fos expression. From

the  $\beta$ -actin levels which were expressed, all of the control measurements were calculated as a ratio of the slot containing the most mRNA, this being assigned as 1 (see column of Table A.1). This level of c-fos (0-3) was then multiplied by the inverse of the ratio of ( $\beta$ -actin) mRNA contained in the slot. This resulted in values which were directly comparable for inflammation- and drug-induced changes in c-fos expression.

In this short study there was no time permitted to construct a calibration curve for the relationship between pixel levels and silver grain density (this could have been done with the use of commercial standard strips which emit a known amount of radioactivity). Therefore it should be noted that a linear relationship has been presumed, though further experiments would be needed to ascertain the correct nature of this relationship.

### **A.3. RESULTS**

The values which are quoted are those from Table A.1., these are comparative levels of c-fos mRNA expression (calculated with consideration to the amount of control  $\beta$ -actin contained in the relevant slot).

#### **A.3.1. CONTROLS**

##### **(a) mRNA content - $\beta$ -actin expression**

In order to ensure comparability in the c-fos blots the membrane was stripped of radiolabel and then re-examined for the expression of  $\beta$ -actin, a gene which is considered to be expressed equally throughout cells regardless of treatment (Iadorola, 1988a). The levels of  $\beta$ -actin which were detected can be seen in Figure A.3. They were not entirely uniform, but did show that every slot contained a more than detectable level of mRNA. Table A.3. shows the pixel counts in each autoradiograph slot, as a ratio of the maximum amount of mRNA detected. The slots which contained the control tissue appear to have far more bound mRNA than the others.

##### **(b) Vehicle Control**

Four hourly injections of saline were administered via an indwelling intrathecal cannula in order to evaluate the effect the experimental procedure had on the expression of c-fos mRNA. Unfortunately, technical problems meant that the results obtained were not adequate in this pilot study.

**Figure A.3.**

These are photographic images of examples of the slot-blot autoradiographs. The top photograph represents the control assay, in which  $\beta$ -actin mRNA was detected. Adequate levels of mRNA can be seen in all slots. The lower photograph is from the test assay, showing the very poor detection of c-fos mRNA in all slots.

The table below describes the treatment received by the tissue in the appropriate slot shown opposite.

CFA/no cannula	L-AP3	(S)-CHPG	(S)-CHPG
(S)-CHPG	(S)-CHPG	(S)-CHPG	(S)-CHPG
(S)-CHPG	(S)-CHPG	L-AP3	(no tissue)
L-AP3	L-AP3	L-AP3	CFA/cannula
L-AP3	L-AP3	(S)-CHPG	(S)-CHPG
(S)-CHPG	no CFA/no cannula	no CFA/cannula	L-AP3



**Table A.3.**

This is a summary of the mean results obtained from the autoradiographs in Figure A3 following c-fos and  $\beta$ -actin detection. A rating was assigned to each of the slots containing c-fos mRNA, based on the experimenter's visual assessment of the autoradiograph. Following this, each rating was multiplied by the inverse of the proportion of control  $\beta$ -actin mRNA detected in the slot. The numbers in the final column are appropriate for the assessment of drug-induced changes in c-fos expression.

<b>Condition</b>	<b>n</b>	<b>c-fos score</b>	<b><math>\beta</math>-actin ratio</b>	<b>1/<math>\beta</math>-actin ratio</b>	<b>1/<math>\beta</math>-actin ratio x c-fos</b>
CFA, no cannula	1	3	1	1	3
CFA, cannula	1	1	0.73	1.37	1.37
no CFA, no cannula	1	1	0.86	1.16	1.16
no CFA, cannula	1	0	0.72	1.39	0
(S)-CHPG	11	1.7	0.53	1.89	3.21
L-AP3	8	1.5	0.56	1.79	2.69



**(c) CFA-induced c-fos expression**

Tissue from a rat which did not receive a CFA injection, but was left for 4 hours with an in-dwelling cannula, showed no induced c-fos expression (slot 22; Figure A.3. and Table A.3.). A rat which did not receive either a CFA injection or any back surgery showed an expression level of 1.16, a small rise compared to the previous control.

The level of c-fos mRNA expressed following a CFA injection can be seen in slot 1 (Figure A.3. and Table A.3.). This treatment, 4 hours post-CFA injection resulted an apparent inflammation with a 30-50% increase in paw circumference. This CFA-evoked inflammation seemed to induce a clear rise in the level of c-fos mRNA detected from the spinal cord, with the adjusted measurement being 3. However, the tissue from an animal which also had an in-dwelling cannula did not appear to contain as much detectable c-fos (slot 15, Figure A.3. and Table A.3.), with the level being only 1.37. Further replication of samples and administration of experimental procedures would be required before any conclusions could be drawn from studies like this. Nevertheless, values were obtained from a number of drug-treated samples before shortage of time prevented any further experiments from being carried out.

**A.3.2. METABOTROPIC GLUTAMATE RECEPTOR ANTAGONISTS**

Tissue from rats which had received intrathecally applied (S)-CHPG expressed a mean level of c-fos mRNA of 3.21 (n=11). The mean level of c-fos expression from tissue which had had L-AP3 applied was 3.21 (n=8). Clearly, these values were consistent with the idea that CFA-induced increases in c-fos mRNA expression were not markedly diminished by the mGluR antagonists. This could reflect inadequate access or an authentic lack of involvement.

Nevertheless, these preliminary observations can only be considered as a technical trial, requiring improvements in c-fos mRNA hybridisation/detection conditions and an adequate usage of both control and experimental samples, before any valid conclusions could be drawn.

#### A.4. DISCUSSION

A number of problems arose in this pilot study. Firstly, insufficient controls were available since a lot of tissue was lost during initial experiments, mainly at the mRNA-purification stage. This loss of data was increased due to problems which arose in the process of developing the technique and optimising conditions for the assay itself. There was inadequate time left at the end to repeat the control experiments in which much tissue had initially been lost. Furthermore, although it was relatively convenient for processing a large number of samples the slot blot technique appeared to be sufficiently sensitive only when there were large amounts of the target mRNA present, i.e. in the case of  $\beta$ -actin, but not c-fos. Apart from technical improvements increased levels of c-fos expression could have perhaps have been accomplished with a more prolonged stimulus, such as 6-8 hours of CFA-induced inflammation. Lantéri-Minet *et al* (1993b) observed maximal Fos expression in the superficial dorsal horn after only 4 hours, using immunocytochemistry. Although the aim of this study was to investigate in a simple fashion the effect of mGluR antagonists on c-fos expression in a population of cells, immunocytochemistry or *in situ* hybridisation histochemistry (ISHH) allowing the detection of c-fos in individual dorsal horn neurons would have perhaps been more successful. This would overcome the signal to noise problem undoubtedly associated with attempts to measure responses to a relatively small number of cells in combined extracts from both these and a large number of unresponsive cells. ISHH was employed by another member of our laboratory (F.E. Munro PhD thesis, 1995) to observe the effect of cutaneously applied mustard oil on c-fos mRNA expression. Levels of c-fos mRNA were observed to increase significantly on the side ipsilateral to the administration of the irritant, after only 45 minutes. This evidence, along with that from Lantéri-Minet *et al* (1993b), would imply that there should be significant levels of c-fos expression in the spinal cord with the current model, and therefore the difficulties experienced in these pilot experiments are primarily of a technical nature. Therefore, since these initial experiments time has been spent, along with another colleague, on refining this protocol. The use of a more sensitive hyperfilm and a wider range of stimulus-response times may well lead to a substantial improvement in the results.

## **APPENDIX 2:**

### **Publications arising from research**

Some of the work from this Thesis have been published:

1. Fleetwood-Walker S.M., Parker R.M.C. Munro F.E., Young M.R. & Mitchell R. (1993) The role of NK<sub>1</sub> and NK<sub>2</sub> receptors in nociceptive inputs to dorsal horn lamina I and laminae IV/V neurons, *Neuropeptides* **24(4)** P82
2. Fleetwood-Walker S.M., Parker R.M.C., Munro F.E., Young M.R. & Mitchell R. (1993) Evidence for the involvement of NK<sub>2</sub> rather than NK<sub>1</sub> receptors in both acute and sustained nociceptive inputs to dorsal horn neurons, Congress Abstracts, XXXII Congress of the International Union of Physiological Sciences 299.6/0.
3. Fleetwood-Walker S.M., Parker R.M.C., Munro F.E. Young M.R. & Mitchell R. (1993) Evidence for a role NK<sub>2</sub> receptors in mediating acute and sustained nociceptive inputs to dorsal horn neurons, Congress Abstracts, IASP Publications, 7th World Congress on Pain, 11 (A23)
4. Fleetwood-Walker S.M., Parker R.M.C., Munro F.E., Young M.R., Hope P.J. & Mitchell R. (1993) Evidence for a role for tachykinin NK<sub>2</sub> receptors in mediating brief nociceptive inputs to rat dorsal horn (laminae III - V) neurons, *European Journal of Pharmacology* **242**: 173-181
5. Munro F.E., Young M.R., Fleetwood-Walker S.M., Parker R.M.C. & Mitchell R. (1993) Receptor and cellular mechanisms involved in mustard oil - induced activation of dorsal horn neurons, Congress Abstracts, IASP Publications, 7th World Congress on Pain, 467 (A1259)

6. Munro F.E., Young M.R., Fleetwood-Walker S.M., Parker R.M.C. & Mitchell R., Receptor and cellular mechanisms involved in mustard oil - induced activation of dorsal horn neurons. in *Progress in Pain Research & Management Vol 2: Proceedings of the 7th World Congress on Pain*. Eds Gebhart GF, Hammond DL & Jensen TS, 337-346
  
7. Fleetwood-Walker S.M., Parker R.M.C., Munro F.E., Young M.R., Hope P.J. & Mitchell R. (1994) The role of tachykinins and somatostatin in spinal nociceptive processing, *The Journal of the Pain Society*, **11** (1): 22-26
  
8. Young M.R., Fleetwood-Walker S.M., Munro F.E. & Mitchell R. (1994) Evidence for a role of metabotropic glutamate receptors in sustained nociceptive inputs to rat dorsal horn neurons, *Neuropharmacology* **33**: 141-144
  
9. Young M.R., Fleetwood-Walker S.M., Birch P.J., Bountra C. & Bowers J. (1995) The role of neurokinin and metabotropic glutamate receptors in thermal and mechanical paw withdrawal responses in normal and carrageenan-treated rats, *British Journal of Pharmacology* **114**:316P
  
10. Young M.R., Fleetwood-Walker S.M., Mitchell R. & Dickinson T. (1995) The involvement of metabotropic glutamate receptors and their intracellular signalling pathways in sustained nociceptive transmission in rat dorsal horn neurons. *Neuropharmacology* **34**:1033-1041
  
11. Young M.R., Fleetwood-Walker S.M., Birch P.J., Bountra C. & Bowers J. (1995) The interaction between neurokinin<sub>1</sub> and metabotropic glutamate receptors in behavioural nociceptive tests in normal and carrageenan-treated rats. *Society for Neuroscience Abstracts* 554.2

See overleaf

## **P82 The Role of NK<sub>1</sub> and NK<sub>2</sub> Receptors in Nociceptive Inputs to Rat Dorsal Horn Lamina I and Laminae IV/V Neurons**

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Single neurons of chloralose/urethane anaesthetised rats were extracellularly-recorded and drugs applied by iontophoresis.<sup>1</sup> In lamina I, [Met-OMe<sup>11</sup>]SP (NK<sub>1</sub> agonist) had no effect in 9/10 cells whereas NKA and GR 64349 (NK<sub>2</sub>) produced a marked but transient excitation in nearly all cases. However, in laminae IV/V, [Ac-Arg<sup>6</sup>, Sar<sup>9</sup>, Met(O<sub>2</sub><sup>11</sup>)SP<sub>6-11</sub>](NK<sub>1</sub>), NKA and GR 64349 were all effective excitants. In experiments with antagonists on laminae IV/V neurons, L-659874 (NK<sub>2</sub>) selectively inhibited brief thermal nociceptive responses in 9/9 cases but L-668169 (NK<sub>1</sub>) had no effect in 7/9. Similar results were obtained in models of sustained neuronal activation by mustard oil<sup>2</sup> or induction of preprodynorphin mRNA by intraplantar carrageenan.<sup>3</sup> Our results suggest that NK<sub>2</sub> but not NK<sub>1</sub> receptors may be important in nociceptive responses of laminae I and IV/V neurons to brief thermal and modest inflammatory stimuli. NK<sub>1</sub> receptors may be more important in profound inflammatory conditions, since in contrast to NKA, SP is released only by damaging levels of thermal stimuli.

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299.6/O

**EVIDENCE FOR THE INVOLVEMENT OF NK<sub>2</sub> RATHER THAN NK<sub>1</sub> RECEPTORS IN BOTH ACUTE AND SUSTAINED NOCICEPTIVE INPUTS TO DORSAL HORN NEURONS**

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Both substance P (SP) and neurokinin A (NKA) are present in fine somatosensory afferents and can be released into spinal dorsal horn by noxious cutaneous stimuli. However, for thermal stimuli, detectable release of SP is not measured until skin temperatures reach levels likely to cause inflammatory damage. Here we assessed the role of NK<sub>1</sub>/NK<sub>2</sub> receptors in acute and sustained (inflammatory) nociceptive inputs to dorsal horn neurons, using iontophoresis of NK receptor drugs. In acute experiments, the NK<sub>2</sub> antagonist L-659874 but not L-668169 (NK<sub>1</sub>) inhibited thermal nociceptive responses of extracellularly-recorded cells without modifying responses to pinch or brush. Agonists for both NK<sub>1</sub> and NK<sub>2</sub> receptors activated laminae IV/V neurons however, suggesting that under some circumstances SP is likely to play some role in nociception. Interestingly, only NK<sub>2</sub> and not NK<sub>1</sub> agonists excited lamina I neurons. The sustained activity of laminae IV/V neurons 'wound-up' by repeated application of mustard oil to the periphery (over 10-30 min) was inhibited by L-659874 but not L-668169 or GR 82334 (NK<sub>1</sub>). Neuronal activation elicited by intraplantar carrageenan (6 h) was assessed by in situ hybridisation for preprodynorphin (PPD) mRNA. The increased expression of PPD mRNA in superficial dorsal horn neurons was inhibited by L-659874 but not L-668169 or GR 82334. It is clear that NK<sub>2</sub> receptors are important in acute thermal nociception and in sustained inflammatory nociception of moderate intensity. It is not clear whether NK<sub>1</sub> receptors may be of greater importance in more profound inflammatory states.

Aug. 23, Monday  
Abstract 23

Room 62 AB  
Slide 3:45 pm

EVIDENCE FOR A ROLE OF NK<sub>2</sub> RECEPTORS IN MEDIATING ACUTE AND SUSTAINED NOCICEPTIVE INPUTS TO DORSAL HORN NEURONS. S. Fleetwood-Walker, R. Parker, F. Munro, M. Young and R. Mitchell (SPON: A. Iggo), Dept. of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH and \*MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ.

Aim of Investigation: In view of reports that tachykinins with selectivity for NK<sub>1</sub> and NK<sub>2</sub> receptors (substance P and neurokinin A respectively) are contained in fine somatosensory afferents and can be released upon noxious stimulation, we assessed the role of these receptors in nociceptive processing.

Methods: The activation of single dorsal horn neurons during cutaneous stimulation was investigated in two models (i) acute natural stimuli to the peripheral receptive field (over 10-30 s) and (ii) peripheral inflammation induced by intraplantar injection of carrageenan (over 6 h). Experiments were carried out in anaesthetised rats. Neuronal responses were measured in model (i) by extracellular electrical recording and in model (ii) by in situ hybridisation histochemistry for preprodynorphin (PPD) mRNA. Antagonists selective for NK<sub>1</sub> or NK<sub>2</sub> receptors were delivered by iontophoresis into the spinal dorsal horn.

Results: Acute sensory responses to noxious heat but not innocuous brush or noxious pinch were inhibited by the NK<sub>2</sub> antagonist L-659874 (9/9 cells) but not by L-668169 (NK<sub>1</sub>; no effect in 7/9 cells), confirming earlier work in cats. In 3 out of 3 animals with bilateral carrageenan inflammation, unilateral L-659874 inhibited the increased expression of PPD mRNA in ipsilateral superficial dorsal horn whereas L-668169 or GR 82334 were ineffective.

Conclusion: These results indicate that dorsal horn NK<sub>2</sub> receptors play a more important role than NK<sub>1</sub> in the present models of acute and sustained nociception. However, since laminae IV/V (although not lamina I) cells are excited by NK<sub>1</sub> as well as NK<sub>2</sub> agonists it seems likely that NK<sub>1</sub> receptors do play some role in nociceptive processing, perhaps under circumstances of more profound inflammation than assessed here.

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## Evidence for a role of tachykinin NK<sub>2</sub> receptors in mediating brief nociceptive inputs to rat dorsal horn (laminae III-V) neurons

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## Evidence for a role of tachykinin NK<sub>2</sub> receptors in mediating brief nociceptive inputs to rat dorsal horn (laminae III–V) neurons

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Since the NK<sub>2</sub> receptor-selective tachykinin, neurokinin A is present in fine primary afferent neurons in addition to the NK<sub>1</sub> receptor-selective tachykinin, substance P, we have addressed the relative role of NK<sub>1</sub> and NK<sub>2</sub> receptors in somatosensory processing in spinal dorsal horn. Recording extracellularly from rat laminae III–V neurons whilst ionophoresing drugs nearby, the selective NK<sub>1</sub> receptor antagonists L 688,169, GR 82334 and [D-Pro<sup>4</sup>,D-Trp<sup>7,9,10</sup>Phe<sup>11</sup>]substance P-(4–11) failed to influence neuronal responses to cutaneous pinch or noxious heat but often enhanced responses to innocuous brush. In contrast, the highly selective NK<sub>2</sub> receptor antagonist L 659,874 profoundly inhibited responses to noxious heat but not pinch or brush. Highly selective synthetic agonists for both NK<sub>1</sub> and NK<sub>2</sub> receptors ([N-acetyl-Arg<sup>6</sup>,Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-(6–11) and GR 64349, respectively) and also NKA showed the inverse effects on sensory responses to those brought about by their antagonists. At higher ionophoretic currents, both NK<sub>1</sub> and NK<sub>2</sub> receptor agonists increased spontaneous activity. This increased basal firing induced by GR 64349 and neurokinin A (but not that due to [N-acetyl-Arg<sup>6</sup>,Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-(6–11) appeared to partially pre-empt further excitatory responses to noxious heat. It is concluded that although both NK<sub>1</sub> and NK<sub>2</sub> receptors can clearly mediate excitation of dorsal horn neurons, it is not NK<sub>1</sub>, but rather NK<sub>2</sub> receptors that are important as the physiological transducer of brief thermal nociceptive inputs in this model.

Tachykinins; Neurokinins; Pain; Analgesia; Spinal cord

### 1. Introduction

The tachykinins substance P and neurokinin A (which display selectivity for NK<sub>1</sub> and NK<sub>2</sub> receptors respectively) are both present in fine somatosensory afferents (Hökefelt et al., 1975; Sundler et al., 1985) and for some time substance P has been a candidate transmitter of nociception (Henry, 1976). There is considerable evidence from electrophysiological, behavioural, biochemical and transmitter release experiments that substance P is involved in nociception (see Fleetwood-Walker and Mitchell, 1989, for review). Nevertheless, the crucial experiments with antagonists have provided mixed results. Early substance P antagonists [D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>]substance P and [D-Pro<sup>2</sup>,D-Phe<sup>7</sup>,D-Trp<sup>9</sup>]substance P failed to reliably reverse the excita-

tory effects of substance P on trigeminal neurons (Hill et al., 1985) and [D-Arg<sup>1</sup>,D-Trp<sup>7,9</sup>Leu<sup>11</sup>]substance P (spantide) failed to antagonise the substance P facilitation of the nociceptive flexor reflex in vivo (Wiesenfeld-Hallin and Duranti, 1987) and only inhibited nociceptive reflexes in vitro at rather high concentrations (Otsuka and Yanagisawa, 1988; Brugger et al., 1990). However, recent NK<sub>1</sub> receptor antagonists spantide II [D-NicLys<sup>1</sup>,3-Pal<sup>3</sup>,D-Cl<sub>2</sub>Phe<sup>5</sup>,Asn<sup>6</sup>,D-Trp<sup>7,9</sup>,Nle<sup>11</sup>]substance P (Maggi et al., 1991b) and CP 96,345 (Snider et al., 1991) have been described to block the facilitation of the flexor reflex and the excitation of dorsal horn neurons brought about by electrical stimulation of afferents at C-fibre intensity (Wiesenfeld-Hallin et al., 1990; De Koninck and Henry, 1991). In behavioural reflex experiments only certain of the early substance P antagonists effective elsewhere on smooth muscle preparations, elevated response thresholds to nociceptive stimuli (Post and Folkers, 1985; Piercey et al., 1986), although several newer NK<sub>1</sub> receptor antagonists were effective in models of inflammatory pain (Yamamoto and Yaksh, 1991; Murray et al., 1991;

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Garret et al., 1991; Birch et al., 1992; Boyce et al., 1992).

Little has been done to address any possible role of NK<sub>2</sub> receptors in dorsal horn, but ionophoretic administration of two early NK<sub>2</sub> receptor antagonists [D-Pro<sup>4</sup>,Lys<sup>6</sup>,D-Trp<sup>7,9,10</sup>,Phe<sup>11</sup>]substance P-(4-11) and [D-Tyr<sup>4</sup>,D-Trp<sup>7,9</sup>,Nle<sup>11</sup>]substance P-(4-11) (Regoli et al., 1985) but not an NK<sub>1</sub> receptor antagonist, spantide, into the substantia gelatinosa, specifically inhibited the responses of cat dorsal horn neurons to noxious thermal, but not other, cutaneous stimuli (Fleetwood-Walker et al., 1990).

Whilst several reports have described direct excitatory effects of substance P on dorsal horn neurons (Henry, 1976; Murase et al., 1989; Urban and Dray, 1992), not only the selective NK<sub>1</sub> receptor agonist [Met-OMe<sup>11</sup>]substance P (Fleetwood-Walker et al., 1990) but also the NK<sub>2</sub> receptor agonist neurokinin A (Salter and Henry, 1991) have been reported to excite dorsal horn lamina IV/V neurons when ionophoretically ejected nearby.

The present experiments were carried out with a range of recently developed agonists and antagonists which are highly selective for NK<sub>1</sub> and NK<sub>2</sub> receptors in order to further address their relative contribution to mediating spinal nociceptive transmission.

## 2. Materials and methods

### 2.1. General

Experiments were carried out, broadly as described previously (Fleetwood-Walker et al., 1991), on male rats (270–350 g), anaesthetised with intravenous  $\alpha$ -chloralose (60 mg kg<sup>-1</sup>) and urethane (1.2 mg kg<sup>-1</sup>), after induction with halothane. Supplementary doses of  $\alpha$ -chloralose were given as required. Core temperature was maintained at 37–38°C with a thermostatically controlled heated blanket, and in the majority of experiments carotid blood pressure was monitored throughout the experiment. Oxygen (0.1 l min<sup>-1</sup>) was passed over the end of the tracheal cannula to enrich the inspired air. The thoraco-lumbar spinal column was supported by three pairs of clamps. A laminectomy (segments L1–L4) was then carried out and agar injected under the most rostral clamped vertebra and then over the whole area of the laminectomy. A core of agar was removed from above the recording region, the dura carefully cut and a pool of liquid paraffin applied to the region.

### 2.2. Electrophysiological methods and ionophoresis

Extracellular recordings were made via the central barrel (4 M NaCl, pH 4.0–4.5) of a 7-barrelled glass

microelectrode. Electrode tip sizes were 4.0–4.5  $\mu$ m and DC resistances were 5–8 M $\Omega$ . The band-width of the recording amplifier was 1 Hz–7 kHz. One side barrel contained 1 M NaCl (pH 4.0–4.5) for automatic current balancing and current controls (Neurophore Ionophoresis System, Medical Systems Corporation). Another side barrel contained Pontamine Sky Blue dye (2% in 0.5 M sodium acetate for marking recording sites by ejection for 100  $\mu$ A min). The locations of recording sites in laminae III–V verified histologically in 15  $\mu$ m cryostat sections lightly counterstained with neutral red. Other barrels contained the following neurokinin receptor agonists and antagonists: neurokinin A; L 659,874 (acetyl-Leu, Met, Gln, Trp, Phe-NH<sub>2</sub>), L 668,169 (cyclo(Gln, D-Trp, Me-Phe, (R)Gly[ANC-2]Leu, Met)<sub>2</sub>), L 659,877 (cyclo(Gln, Trp, Phe, Gly, Leu, Met)) (all from Cambridge Research Biochemicals); [D-Pro<sup>4</sup>,D-Trp<sup>7,9,10</sup>,Phe<sup>11</sup>]substance P-(4-11) (from Bachem UK); GR 82334 ([D-Pro<sup>9</sup>[spiro- $\gamma$ -lactam]-Leu<sup>10</sup>,Trp<sup>11</sup>]physalaemin-(1-11) and GR 64349 ([Lys<sup>3</sup>,Gly<sup>8</sup>-R- $\gamma$ -lactam-Leu<sup>9</sup>]neurokinin-(3-10) (gifts from Glaxo Group Research) and [N-acetyl-Arg<sup>6</sup>,Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-(6-11) (a gift from Professor D. Regoli). Solutions were either 1 mM in distilled water (GR 82334, neurokinin A and GR 64349) or 0.1 mM in 0.3% dimethylformamide, diluted from concentrated stocks in pure dimethylformamide (other compounds) and were adjusted to pH 4.5 with HCl. All peptide solutions were either freshly made or stored at -20°C in small (100  $\mu$ l) aliquots which once thawed were not refrozen, or used again. All drugs were applied with cathodal currents. Retaining currents of -10 nA were used to minimise drug leakage between tests. Action potentials of the recorded cells were clearly discriminated from other field potentials throughout the test. Neuronal firing was recorded on FM tape (Racal) and firing rates were plotted on-line by computer (IBM PS/2-70-121) together with stimulator and ionophoresis markers.

All the neurons recorded were selected as having cutaneous excitatory receptive fields on the hairy skin of the ipsilateral hind limb that were multireceptive (i.e. mediated responses to both noxious and innocuous stimuli). Receptive fields were initially located by brief manual brush/pinch stimuli, prior to characterisation with timed and quantified stimuli, including also noxious heat. Controlled stimuli were applied to adjacent cutaneous areas within the receptive field on the dorsal surface of the paw or the hind limb were regularly repeated over 3–4 min cycles. The innocuous stimulus used was a motorised, rotating brush. Noxious stimuli were provided by a calibrated pinch (serrated forceps with a graduated controlled displacement) or a thermocouple-controlled radiant heat lamp (giving a skin surface temperature ramp of 30–48°C). Like the other stimuli, the noxious heat stimulus was set routinely for



10 s at 48°C, but in occasional examples for 15 s to ensure a more robust response. Skin temperatures never exceeded the 45°C definition of noxious levels (Duggan et al., 1988) for longer than 1.5 s beyond the 10 or 15 s duration of the pre-set 48°C stimulus. The marker bars in the figures indicate the period of skin temperature in excess of 45°C. The cycle of stimuli was repeated every 3 min, with the ejection of the drugs initiated 1 min before the start of a cycle and continuing through that cycle. The different responses and epochs of spontaneous activity prior to each cycle of stimuli were integrated over 10–15 s periods as appropriate and compared to pre-drug control values, measured from at least two consecutive tests. The stimulus-evoked responses were always submaximal and approximately matched in terms of neuronal firing rates. Duplicate or triplicate control responses were required to vary by less than 15–20%.

### 3. Results

The present results were obtained from a total of 61 neurons which were all located in laminae III–V of the dorsal horn. Peptide antagonists highly selective for NK<sub>1</sub> and NK<sub>2</sub> receptors (L 668,169/GR 82334/[D-Pro<sup>4</sup>,D-Trp<sup>7,9,10</sup>Phe<sub>11</sub>]substance P-(4–11) and L 659,874, respectively; Regoli et al., 1987; McKnight et al., 1988; Hagan et al., 1991) were tested by ionophoresis close to the recorded cells. The vehicle (0.3% dimethylformamide in water) had no effect on 3 out of 3 neurons when ejected at up to 80 nA for 12 min. The rather more potent cyclic analogue of L 659,874, L 659,877, could not be tested because of its low solubility in acceptable vehicles.

Fig. 1 shows typical results (displayed as raw activity records) obtained with the NK<sub>1</sub> receptor antagonist L 668,169 in 6 out of 8 neurons tested. There was little effect of this antagonist on spontaneous activity or any sensory-evoked response apart from a small but consistent enhancement of activity evoked by innocuous brush (fig. 1, table 2). In the remaining 2 cells there were no detectable changes. In 4 cells where recovery was assessed there was 50% (n = 1) and over 85% (n = 3) reversal of the effect of L 668,169 on brush responses by 4 and 9–24 min respectively, after cessation of ionophoresis. Entirely similar results were obtained with GR 82334 in 5 out of 6 cells, showing a small increase in responses to brush, but no effect on those to noxious heat (fig. 2, Table 2). In 2 neurons tested with [D-Pro<sup>4</sup>,D-Trp<sup>7,9,10</sup>Phe<sub>11</sub>]substance P-(4–11) (30–40 nA, 6 min) brush responses were increased by 33 and 41% with no consistent change in other activity. Ionophoretic currents up to 50, 45 and 80 nA were tested for L 668,169, GR 82334 and [D-Pro<sup>4</sup>,D-Trp<sup>7,9,10</sup>Phe<sub>11</sub>]substance P-(4–11) respectively, until ei-

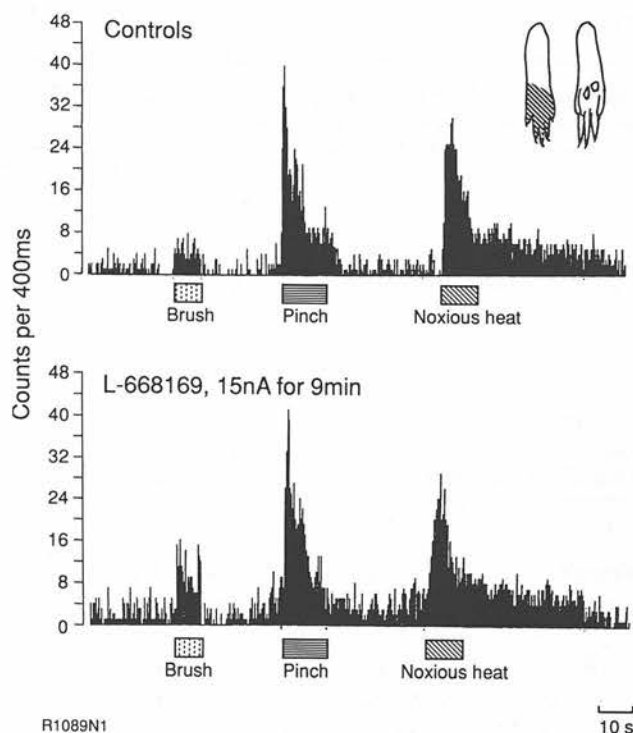


Fig. 1. Ongoing firing frequency records showing typical effects of the NK<sub>1</sub> receptor antagonist L 668,169 on somatosensory responses of a laminae III–V neuron. A modest facilitation of responses to innocuous brush was observed in 6 out of 8 neurons tested, with no other consistent changes. Recovery from drug effects was not examined closely, but in 3 out of 4 cells examined, responses to brush had recovered to within  $\pm 15\%$  of control levels within 9–24 min after stopping ionophoresis of the drug. The fourth cell showed approximately 50% recovery within 4 min. The inset shows the position of the cutaneous receptive field on the ipsilateral hind limb. The duration of each of the stimuli is indicated, in the case of noxious heat, this representing the period for which the skin temperature exceeded the generally accepted noxious threshold of 45°C.

ther spike reduction or technical factors prevented further study. The effects on sensory responses at these currents were similar to those at lower currents, notably there still being no consistent inhibition of responses to noxious heat.

The NK<sub>2</sub> receptor antagonist L 659,874 produced a quite different result: a highly selective inhibition of responses to noxious thermal stimuli without altering responses to noxious pinch, innocuous brush or spontaneous activity (fig. 3, table 2). This was seen in 8 out of 10 neurons tested at just 10 nA of L 659,874 (mean 49% inhibition), with no detectable effect in the others. At a higher ejection current (20 nA) the selective inhibition was seen in all cells tested (9 out of 9) and was of a greater degree (mean 66% inhibition) (this was particularly clear in 4 cases where both currents were tested on the same cell). In 3 neurons where recovery was investigated, the inhibition of heat responses by L 659,874 had partially recovered (to 50–85% of controls) within 6–15 min after cessation of

TABLE 1

Time dependence of the effects of neurokinin receptor antagonists on thermal nociceptive responses.

All values represent evoked activity (corrected for background) and are shown as percentages of the mean stimulus-induced activity occurring during the stimulus. Values are expressed as mean  $\pm$  S.E.M.

	Noxious heat-induced increase in neuronal firing rate (% of control response during stimulus)		
	During stimulus	0–10 s after stimulus	10–20 s after stimulus
<i>(a) L 668,169 (n = 6)</i>			
Control	100 (11 $\pm$ 1 s)	21 $\pm$ 7	15 $\pm$ 9
Drug (20 nA for 6 min)	90 $\pm$ 6	20 $\pm$ 11	9 $\pm$ 7
<i>(b) GR 82334 (n = 5)</i>			
Control	100 (10 s)	28 $\pm$ 7	9 $\pm$ 5
Drug (32 $\pm$ 4 nA for 6 min)	93 $\pm$ 10	23 $\pm$ 12	8 $\pm$ 7
<i>(c) L 659,874 (n = 9)</i>			
Control	100 (11 $\pm$ 1 s)	25 $\pm$ 9	11 $\pm$ 9
Drug (20 nA for 6 min)	36 $\pm$ 6	10 $\pm$ 6	7 $\pm$ 7

iontophoresis. In the majority of cells tested here, responses to noxious heat declined rapidly on cessation of the stimulus (to less than 20% of the response during the stimulus, within the subsequent 10 s). For each of the drugs tested there were, however, examples of relatively prolonged after discharges, retaining around 50% of the peak activity during the first post-stimulus period. There was no evidence that effects of any of the drugs were greater (or in any way different) in post-stimulus periods than during application of the heat stimulus (table 1).

A number of NK<sub>1</sub> and NK<sub>2</sub> receptor agonists were also examined (table 2). The highly selective NK<sub>1</sub> receptor agonist [N-acetyl-Arg<sup>6</sup>,Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-(6–11) (Regoli et al., 1988) was tested either

on neuronal responses to cycled cutaneous stimuli either at low or higher iontophoretic currents. In 9 out of 13 cells [N-acetyl-Arg<sup>6</sup>,Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-(6–11) (when ejected at relatively low currents: mean 31  $\pm$  4 nA) caused an inhibition of the activity evoked by innocuous brush with no apparent effect on spontaneous activity or nociceptive responses. In 6 out of 6 cells, higher ejection currents (mean 57  $\pm$  5 nA) of [N-acetyl-Arg<sup>6</sup>,Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-(6–11) increased spontaneous activity, causing marked and sustained rises to around 5-fold of pre-drug controls. Recovery was observed in all 5 cases examined within 5–10 min after terminating iontophoresis and the drug effect was not replicated in current (NaCl) or vehicle control tests.

TABLE 2

Summary of the effects of neurokinin receptor antagonists and agonists on laminae III–V neurons.

All values are calculated as a percentage of the mean pre-drug control value and are expressed as the mean  $\pm$  S.E.M. Responses were designated as being essentially unchanged if they remained within 20% of the mean control value. Neurons in the population tested which were not influenced in the characteristic predominant fashion illustrated, all showed no (or no consistent) changes in their responses. For the subpopulations of neurons affected by the drugs, the statistical significance of changes was assessed by Wilcoxon test.

Drug and conditions		Stimulus-evoked neuronal firing rate (% of mean pre-drug control)					
		Spontaneous	Brush	Pinch	Noxious heat	Number of neurons influenced	Number of neurons tested
<i>Antagonists</i>							
L 668,169 (NK <sub>1</sub> )	20 nA for 6 min	95 $\pm$ 7	141 $\pm$ 9 <sup>a</sup>	105 $\pm$ 10	82 $\pm$ 9	6	8
GR 82334 (NK <sub>1</sub> )	32 $\pm$ 4 nA for 6 min	103 $\pm$ 8	131 $\pm$ 8 <sup>a</sup>	—	91 $\pm$ 11	5	6
L 659,874 (NK <sub>2</sub> )	10 nA for 6 min	93 $\pm$ 7	103 $\pm$ 9	99 $\pm$ 8	51 $\pm$ 6 <sup>a</sup>	8	10
	20 nA for 6 min	95 $\pm$ 12	104 $\pm$ 6	85 $\pm$ 11	34 $\pm$ 5 <sup>a</sup>	9	9
<i>Agonists</i>							
[Ac-Arg <sup>6</sup> ,Sar <sup>9</sup> ,Met(O <sub>2</sub> ) <sup>11</sup> ]-substance P-(6–11) (NK <sub>1</sub> )							
	31 $\pm$ 4 nA for 5 $\pm$ 1 min	121 $\pm$ 13	52 $\pm$ 5 <sup>a</sup>	98 $\pm$ 5	92 $\pm$ 6	9	13
	57 $\pm$ 5 nA for 6 $\pm$ 2 min	536 $\pm$ 88 <sup>a</sup>	67 $\pm$ 9 <sup>a</sup>	91 $\pm$ 14	106 $\pm$ 11	6	6
Neurokinin A (NK <sub>2</sub> )	11 $\pm$ 5 nA for 7 $\pm$ 1 min	112 $\pm$ 10	89 $\pm$ 11	108 $\pm$ 10	155 $\pm$ 13 <sup>a</sup>	6	6
	44 $\pm$ 4 nA for 6 $\pm$ 1 min	354 $\pm$ 61 <sup>a</sup>	79 $\pm$ 19	120 $\pm$ 21	64 $\pm$ 16 <sup>a</sup>	6	8
GR 64349 (NK <sub>2</sub> )	66 $\pm$ 6 nA for 7 $\pm$ 1 min	398 $\pm$ 54 <sup>a</sup>	111 $\pm$ 13	88 $\pm$ 20	75 $\pm$ 11 <sup>a</sup>	5	5

<sup>a</sup> P < 0.05, compared to mean pre-drug control.

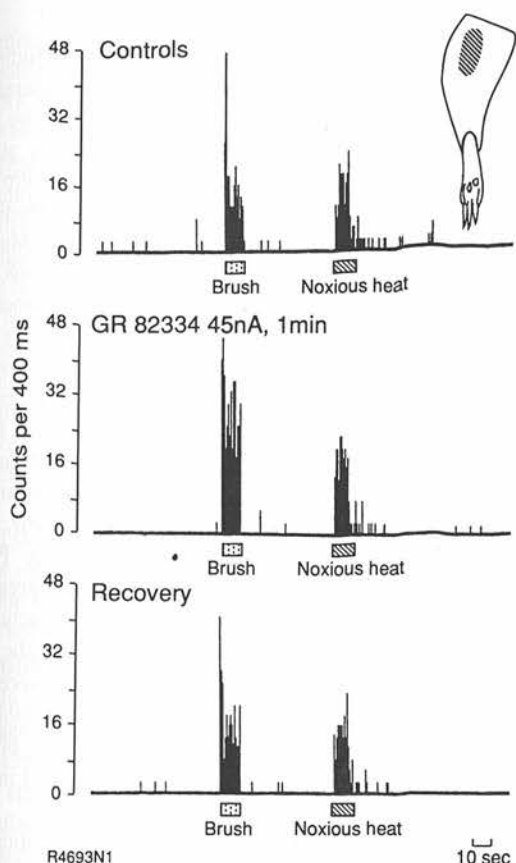


Fig. 2. Ongoing firing frequency records showing typical effects of the  $NK_1$  receptor antagonist GR 82334 on somatosensory responses of a laminae III-V neuron. Facilitation of responses to innocuous brush (with no other consistent change) was observed in 5 out of 6 cells examined. The lower trace shows recovery 3 min after termination of ionophoresis. Similar effects were observed in 3 further cells where recovery was assessed. The inset shows the position of the cutaneous receptive field on the ipsilateral hind limb.

The  $NK_2$  receptor agonists, neurokinin A and the novel highly selective compound GR 64349 (Hagan et al., 1991) also increased spontaneous activity of laminae III-V neurons in a consistent manner. Neurokinin A ( $44 \pm 4$  nA) and GR 64349 ( $66 \pm 6$  nA) produced marked and sustained excitation of spontaneous activity in 6 out of 8 and 5 out of 5 cases respectively. When neurokinin A was tested at low currents (mean  $11 \pm 5$  nA) which did not raise spontaneous activity in the recorded neuron, there was a selective facilitation of the responses to noxious thermal stimuli (in 6 out of 8 cells). Responses to innocuous brush and noxious pinch were essentially unaltered. Although one cell showed a 30% increase in the noxious pinch response, this was not reproduced in the other examples. At the higher ionophoretic currents of neurokinin A and GR 64349 that increased spontaneous activity, noxious heat-evoked responses were attenuated when corrected for the elevated baseline. Other responses were essentially unaltered. In 2 cases with neurokinin A, the increment in spontaneous activity was equivalent to the magni-

tude of reduction in heat responses. In all other cases this magnitude of reduction was greater (mean 3–9-fold) than the actual increase in spontaneous activity. Recovery was observed within 6–12 min after terminating ionophoresis and none of the effects was reproduced in current control tests. In 4 cells where [N-acetyl-Arg<sup>6</sup>,Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-(6–11) and neurokinin A were each tested, clear excitatory responses to both the  $NK_1$  and  $NK_2$  receptor agonist were seen.

Fig. 4 (representative of 3 out of 3 neurons) shows the selective facilitation of thermal nociceptive responses by a low current of neurokinin A and a reduction in those facilitated responses when L 659,874 was additionally applied. This is consistent with reversal of the effect of exogenous neurokinin A by the selective  $NK_2$  receptor antagonist but cannot be unequivocal, since the antagonist alone had inhibitory effects on thermal nociceptive responses (fig. 3). Analogous results were obtained in 3 experiments when the effect of GR 82334 was tested on the inhibition of innocuous

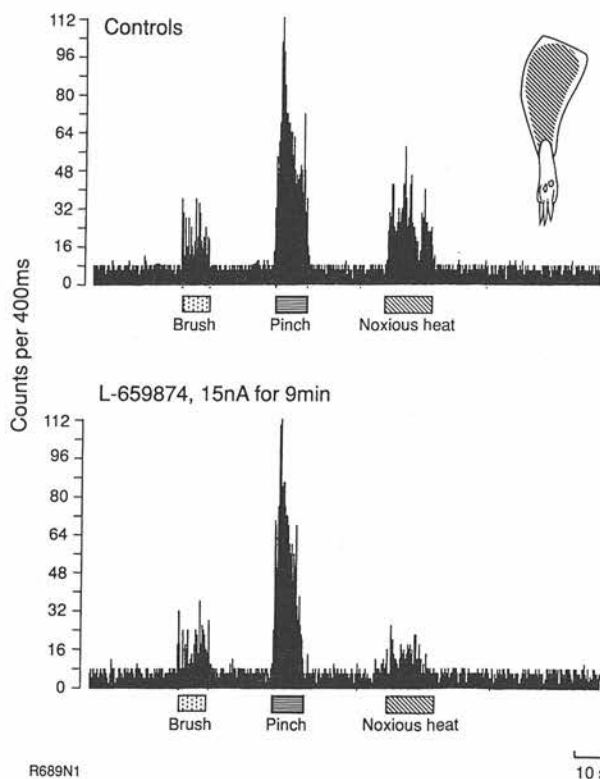


Fig. 3. Ongoing firing frequency records showing typical effects of the  $NK_2$  receptor antagonist L 659,874 on somatosensory responses of a laminae III-V neuron. A marked attenuation of responses to noxious heat but not other stimuli or spontaneous activity was seen in all 11 neurons tested at currents of 15–20 nA. Recovery from drug effects was not examined closely, but in 3 out of 3 cells examined there was recovery of the noxious heat response to within 50–85% of control at 6–15 min after terminating ionophoresis of the drug. The inset shows the position of the cutaneous receptive field on the ipsilateral hind limb.

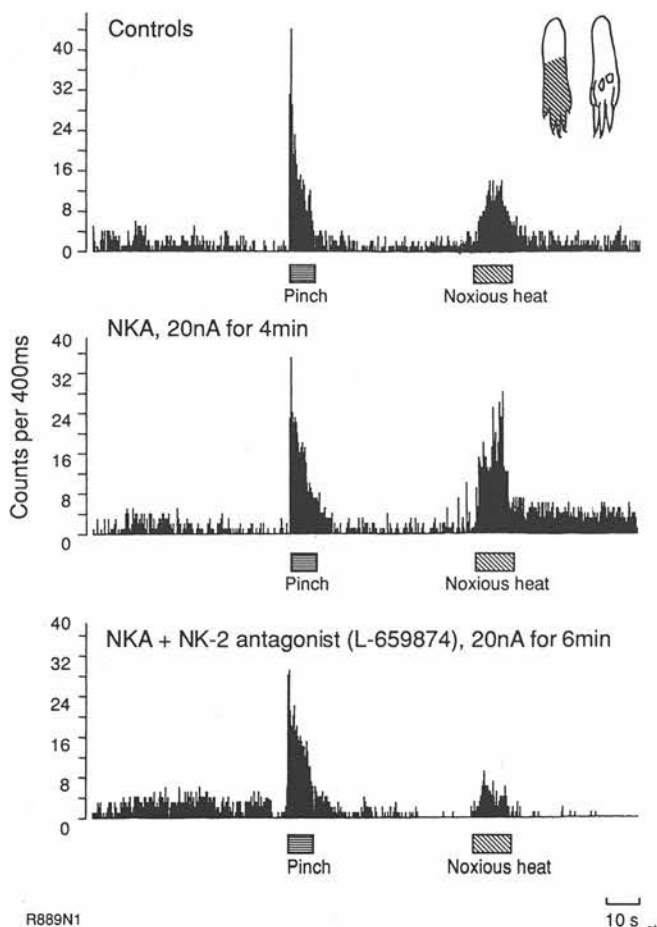


Fig. 4. Ongoing firing frequency records showing typical effects of the NK<sub>2</sub> receptor agonist neurokinin A, in the absence or presence of the NK<sub>2</sub> receptor antagonist L 659,874 on the somatosensory responses of a laminae III–V neuron. Neurokinin A caused a selective facilitation of responses to thermal but not mechanical noxious stimuli. Responses to innocuous brush (not shown here) were also unaltered (see table 2). Results were typical of 6 neurons tested with low currents of neurokinin A. After the middle trace had been recorded, iontophoresis of L 659,874 was begun in addition to neurokinin A. A marked attenuation of the neurokinin A-amplified response to noxious heat to below control levels was observed in all 3 cells so tested. The inset shows the position of the cutaneous receptive field on the ipsilateral hind limb.

brush responses induced by low currents of [N-acetyl-Arg<sup>6</sup>,Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-(6–11). Effects of antagonists on the increases in spontaneous activity brought about by high ejection currents of NK<sub>1</sub> and NK<sub>2</sub> receptor agonists were not investigated in the present study.

#### 4. Discussion

These experiments provided no clear evidence that NK<sub>1</sub> receptors participate in transducing brief nociceptive inputs to laminae III–V neurons of rat spinal dorsal horn under the present conditions. Since the

potency of some NK<sub>1</sub> receptor antagonists, including L 668,169, is reduced in rodent bioassays compared to those in other species (Pattachini et al., 1992), we also tested GR 82334 (which retains high potency at rat NK<sub>1</sub> receptors; Beresford et al., 1992) and [D-Pro<sup>4</sup>,D-Trp<sup>7,9,10</sup>,Phe<sup>11</sup>]substance P-(4–11) (which is effectively antinociceptive in the mouse formalin model; Murray et al., 1991). The NK<sub>1</sub> receptor antagonists L 668,169, GR 82334 and [D-Pro<sup>4</sup>,D-Trp<sup>7,9,10</sup>,Phe<sup>11</sup>]substance P-(4–11) had no significant effect on nociceptive responses in any neurons tested, but in contrast caused a modest facilitation of their responses to innocuous brush in the majority of cells. This suggests that their lack of effect on nociceptive responses was not due to failure to reach relevant sites at effective concentrations. Conviction that this effect of L 668,169, GR 82334, and [D-Pro<sup>4</sup>,D-Trp<sup>7,9,10</sup>,Phe<sup>11</sup>]substance P-(4–11) was due to NK<sub>1</sub> receptor antagonism is strengthened by that being the common property of structurally quite distinct compounds, by the low ionophoretic currents required, by the observation that NK<sub>1</sub> receptor agonists produce precisely the inverse effect (table 2) and by the apparent reduction in the effect of [N-acetyl-Arg<sup>6</sup>,Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>] substance P-(6–11) in the presence of GR 82334.

The simplest interpretation of the facilitation of brush responses by NK<sub>1</sub> receptor antagonists seems to be that NK<sub>1</sub> receptors are responsible for an attenuating influence on non-nociceptive inputs to these cells and that the antagonist is acting to reverse the action of the endogenous NK<sub>1</sub> receptor agonist, substance P, which has been released during the course of our experimental procedure. The factors leading to substance P release here are unclear (see below), but substance P does not appear to be responsible for acutely mediating the increased activity elicited by brief thermal or mechanical noxious stimuli. It is of course possible that the antagonists have unknown side effects or that more complex interactions are occurring. The NK<sub>1</sub> receptor agonist [N-acetyl-Arg<sup>6</sup>,Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>] substance P-(6–11) clearly increased the spontaneous activity of laminae III–V neurons here (table 2), indicating that NK<sub>1</sub> receptors can exert a direct (or at least a general modality-independent) influence on activity of these cells. The data indicating that NK<sub>1</sub> receptor activation can excite these cells, but seems not to participate in nociceptive responses and instead attenuates non-nociceptive tactile responses (in at least a subpopulation of cells) are entirely consistent, in our hands, between rat and cat. Somewhat in contrast to the present results, Radhakrishnan and Henry (1991) reported inhibition by CP-96,345 of the after-discharge following brief noxious mechanical or thermal stimuli. There was little effect on the initial phase of excitatory responses to brief heat stimuli and inconsistent effects on pinch-evoked excitation. It is possible that CP-96,345



is effective only on late components of nociceptive responses or that actions of this compound other than on NK<sub>1</sub> receptors may be involved. Indeed, there is new evidence that CP-96,345 is a potent antagonist of L-type Ca<sup>2+</sup> channels (Schmidt et al., 1992) and furthermore that such compounds are effective antinociceptive agents (Miranda et al., 1992). Although both peptide and non-peptide NK<sub>1</sub> receptor antagonists have been reported to block the late prolonged but not the brief excitatory postsynaptic potentials induced in dorsal horn neurons by C-fibre activation or by noxious stimuli (Urban and Randic, 1984; De Koninck and Henry, 1991), in our experiments, there was no evidence that NK<sub>1</sub> receptor antagonists were any more effective in the 20 s of afterdischarge following a noxious heat stimulus than they were during the response itself (table 1).

In experiments designed to examine adequate stimuli for the release of substance P into spinal cord, it has been reported that noxious pinch, intraplantar formalin, topical methylene chloride and flexion of a kaolin/carrageenan-inflamed knee-joint were effective (Duggan et al., 1988; Kuraishi et al., 1989; Schaible et al., 1990). Thermal cutaneous stimuli, however, were only effective at skin temperatures considered to result in inflammatory cutaneous lesions (Duggan et al., 1988; Kuraishi et al., 1989). In contrast, both noxious mechanical stimuli and noxious thermal stimuli (at skin temperatures below those producing inflammatory damage) were effective in releasing neurokinin A (Duggan et al., 1990). Behavioural reports have described inhibition by CP-96,345 of the second (inflammation-supported) phase of the response to intraplantar formalin (Yamamoto and Yaksh, 1991) and inhibition by [Arg<sup>1</sup>,D-Pro<sup>2</sup>,D-Phe<sup>7</sup>,D-His<sup>9</sup>]substance P of the secondary contralateral hyperalgesia following heat injury (Coderre and Melzack, 1991). Correspondingly, only the facilitation of nociceptive flexor reflex by substance P or C-afferent conditioning stimuli (and not the reflex itself) was inhibited by the NK<sub>1</sub> receptor antagonist spantide II (Wiesenfeld-Hallin et al., 1990). Thus, our evidence and the data from behavioural and release experiments point towards the involvement of substance P in mediating inflammation-supported nociception rather than responses to brief noxious stimuli.

In contrast to the results concerning NK<sub>1</sub> receptors, the NK<sub>2</sub> receptor antagonist L 659,874 caused a marked and highly consistent inhibition of nociceptive responses without affecting spontaneous activity or responses to innocuous brush. Inhibition by the NK<sub>2</sub> receptor antagonist showed a striking selectivity for thermal rather than mechanical nociceptive stimuli, a result also noted in cat with the moderately selective NK<sub>2</sub> receptor antagonist [D-Pro<sup>4</sup>,Lys<sup>6</sup>,D-Trp<sup>7,9,10</sup>,Phe<sup>11</sup>]substance P-(4-11) (Fleetwood-Walker et al., 1990). The mechanistic basis for this is unclear, since

polymodal C-afferents are likely to contribute greatly to both responses. Nevertheless, small dorsal root ganglion cells are very heterogeneous in terms of their neuropeptide content and there is evidence that thermal/mechanical noxious stimuli can differentially elicit release of neuropeptides into spinal perfusates (Kuraishi et al., 1989). Consistent with a role of an NK<sub>2</sub> receptor agonist such as neurokinin A in thermal, but not mechanical nociception is its selective facilitation of thermal nociceptive responses (fig. 4, a result also seen in cat with neurokinin A and other agonists with NK<sub>2</sub> receptor selectively (Fleetwood-Walker et al., 1990). In the current experiments, both neurokinin A and the highly selective NK<sub>2</sub> receptor agonist GR 64349 further caused marked and consistent increases in spontaneous activity of laminae III-V neurons, consistent with the idea that NK<sub>2</sub> receptor activation is an important element in the transduction of thermal nociceptive inputs. It is of course possible that co-factors may also subserve an important role. Interestingly, although both NK<sub>1</sub> and NK<sub>2</sub> receptor agonists increase the spontaneous activity in laminae III-V cells, the situation is quite different in lamina I where only NK<sub>2</sub> but not NK<sub>1</sub> receptor agonists are effective (Fleetwood-Walker et al., 1992).

There is increasing biochemical evidence that NK<sub>2</sub> receptors (although generally at much lower abundance than NK<sub>1</sub> or NK<sub>3</sub> receptor sites) are present in the CNS and in particular in the dorsal horn of the spinal cord (Yashpal et al., 1990; Poosch et al., 1991; Takeda and Krause, 1991). Additionally functional studies from other groups are now beginning to support a role for NK<sub>2</sub> receptors in spinal nociception. Xu et al. (1991) described that the NK<sub>2</sub> receptor antagonist, [Tyr<sup>5</sup>,D-Trp<sup>6,8,9</sup>,Arg<sup>10</sup>]neurokinin A-(4-10) (MEN 10207) selectively reversed the facilitation by neurokinin A (but not substance P) of the spinal nociceptive flexor reflex. Furthermore, a congener with further reduced partial efficacy [Tyr<sup>5</sup>,D-Trp<sup>6,8,9</sup>,Lys<sup>10</sup>]neurokinin A-(4-10) (MEN 10376; Maggi et al., 1991a) inhibited the synaptic excitation of dorsal horn neurons evoked by capsaicin administration to the dorsal root ganglia (but not that evoked by an NK<sub>1</sub> receptor agonist) in a spinal cord slice with attached dorsal roots (Urban et al., 1992).

The pharmacological properties of the small, but functionally important population of NK<sub>2</sub> receptors in dorsal horn remain to be investigated in detail.

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HALL TERNES  
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RECEPTOR AND CELLULAR MECHANISMS INVOLVED IN MUSTARD OIL-INDUCED ACTIVATION OF DORSAL HORN NEURONS. F. Munro, M. Young, S. Fleetwood-Walker, R. Parker and R. Mitchell\* Dept of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH and \*MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ.

Aim of Investigation: The C-fibre-selective chemical irritant mustard oil produces sustained activity of dorsal horn neurons upon repeated peripheral application and long-lasting changes in receptive fields and responsiveness. We addressed the possible role of NK<sub>1</sub>, NK<sub>2</sub> and metabotropic excitatory amino acid receptors and of protein kinase C (PKC) in responses to mustard oil.

Methods: Mustard oil was applied to restricted distal areas of one hindlimb of rats. In some experiments tissue was taken for biochemical assay of the translocation/activation of PKC assessed by movement of [<sup>3</sup>H]phorbol dibutyrate ([<sup>3</sup>H]PDBu) binding sites from cytosolic to membrane fractions. Extracellular recordings of single dorsal horn neurons were made in anaesthetised rats and drugs applied by iontophoresis.

Results: Selective antagonists for NK<sub>2</sub> and metabotropic receptors, L-659874 and L-AP3 reliably inhibited neuronal activity 'wound-up' by repeated application of mustard oil (in 19/21 and 6/9 cases respectively). The NK<sub>1</sub> antagonists, L-668169 and GR 82334 had no effect in 13/17 cases. The selective PKC inhibitors chelerythrine and GF 109203X caused marked, yet recoverable, inhibition of activity in 8/9 and 8/8 cases respectively. In corresponding spinal segments, significantly greater proportions of [<sup>3</sup>H]PDBu binding sites were associated with the membrane fraction on the side ipsilateral to mustard oil application.

Conclusion: Both NK<sub>2</sub> and metabotropic receptors appear to play a role in the sustained activation of dorsal horn neurons elicited by mustard oil. There is good evidence that PKC is a crucial mediator of this cellular activation. NK<sub>1</sub> receptors appear to be of less importance in this model of sustained nociception.

## Receptor and Cellular Mechanisms Involved in Mustard Oil-Induced Activation of Dorsal Horn Neurons

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### INTRODUCTION

For many years substance P (SP) has been a prominent candidate for the transmitter of primary afferent nociceptive fibers (Henry 1976). Although considerable evidence suggests a role for SP (and NK<sub>1</sub> receptors) in nociceptive transmission (Coderre et al. 1993; Maggi et al. 1993), it is unlikely to be the only relevant transmitter. SP together with neurokinin A (NKA) and excitatory amino acids (EAAs) are released into the dorsal horn by noxious or high-intensity stimulation of afferents (Duggan et al. 1990; Skilling et al. 1988; Petermann et al. 1986). Evidence from both electrophysiological and reflex studies suggests that transmitters such as NKA and EAAs also may be crucial to nociception through their actions at NK<sub>2</sub>, NMDA, and non-NMDA receptors (Coderre et al. 1993).

Several lines of evidence point to a role for NKA in nociceptive transmission. Ligand binding (Yashpal et al. 1990) and mRNA hybridization studies (Takeda and Krause 1991) have confirmed that there are NK<sub>2</sub> receptors in the spinal cord. NKA is present in primary sensory neurons (Ogawa et al. 1985) and is released by capsaicin from primary afferent terminals (Saria et al.

1986). In a model of acute nociception, we have previously shown that the responses of dorsal horn neurons to brief thermal (but not mechanical) noxious cutaneous stimuli are inhibited by selective NK<sub>2</sub> but not NK<sub>1</sub> receptor antagonists (Fleetwood-Walker et al. 1990, 1992a). Also in an *in vitro* spinal cord/dorsal root preparation, neuronal activity evoked by brief application of capsaicin to the dorsal root ganglion is inhibited by a selective NK<sub>2</sub> antagonist (Urban et al. 1992). Following both noxious mechanical and noxious thermal stimuli, release of NKA-immunoreactive material is detected throughout the dorsal horn in a diffuse pattern (Duggan et al. 1990; Hope et al. 1990) and intrathecal administration of NKA gives rise to behavioral responses indicative of pain (Cridland and Henry 1986; Fleetwood-Walker et al. 1990).

The EAA glutamate is present at high levels in local interneurons of the dorsal horn (Rizzoli 1968; Fagg and Foster 1983; Storm-Mathisen and Ottersen 1987) as well as in primary afferent terminals, as demonstrated by immunolabeling of granular vesicles following rhizotomy (DeBiasi and Rustioni 1988). Upon noxious cutaneous stimulation or low- or high-intensity electrical stimulation to peripheral nerves, glutamate is released (Skilling et al. 1988), where it may act via several receptors (Schoepp et al. 1990a; Watkins et al. 1990). The ionotropic group of receptors, including  $\alpha$ -amino-3-hydroxy-5-isoxazole-4-propionate (AMPA) and N-methyl-D-aspartate (NMDA) types, have been extensively studied and it is now well established that the NMDA receptor is involved in hypersensitized states such as long-term potentiation (LTP) in the hippocampus (Collingridge 1992) and wind-up in the spinal cord (Davies and Lodge 1987; Dickenson and Sullivan 1987, 1990; Thompson et al. 1990). A metabotropic group of glutamate receptors (mGlu), linked to G-proteins, also may be involved in such transmission. Studies have demonstrated a role for mGlu receptors, including a role in an NMDA receptor-independent model of LTP (Bortolotto and Collingridge 1993). Previous studies have demonstrated that 1-amino-cyclopentane-1,3-dicarboxylic acid (ACPD), a metabotropic glutamate receptor agonist (Irving et al. 1990), can augment the actions of ionotropic agonist-induced activity in spinal cord preparations (Cerne and Randic 1992; Bleakman et al. 1992), although the cellular mechanism responsible has yet to be elucidated.

In our study, we used a prolonged noxious chemical stimulus to investigate the involvement of NK<sub>1</sub>, NK<sub>2</sub>, mGlu receptors, and protein kinase C (PKC) in the sustained activation of dorsal horn neurons. The model used is topical cutaneous application of mustard oil (allyl isothiocyanate), a chemical irritant that selectively activates C (and occasionally some A-delta) afferent fibers (Heapy et al. 1987).



## METHODS

### ELECTROPHYSIOLOGY

We conducted experiments on male Wistar rats (250–340 g) anaesthetized with intravenous  $\alpha$ -chloralose (60 mg/kg) and urethane (1200 mg/kg) after induction with halothane. Supplementary doses of  $\alpha$ -chloralose were given as required. Rectal temperature was continuously monitored and maintained between 37–38°C. The rats respired spontaneously, but oxygen was supplied to the area of the tracheal cannula to enrich the inspired air.

The thoraco-lumbar spinal column was supported by clamps under the lateral processes and a laminectomy (segments L1–L4) was performed. To increase the recording stability, a 2% agar solution was injected under the spinal bone at the rostral end of the laminectomy and then poured over the exposed cord. A core of agar was removed from above the recording region, the dura carefully cut, and a pool of liquid paraffin applied to the region.

Extracellular recordings were made via the central barrel (4 M NaCl pH 4.0–4.5) of a seven-barreled glass microelectrode. One side barrel contained 1 M NaCl (pH 4.0–4.5) for automatic current balancing and current controls. Another side barrel contained Pontamine Sky Blue (2% in 0.5 M sodium acetate) for determination of neuronal location by ejection at the recording site followed by histological examination. The other barrels of the electrode contained NK<sub>1</sub>, NK<sub>2</sub> or mGlu receptor antagonists or PKC inhibitors, which were applied locally by iontophoresis over a range of currents (10–80 nA) for several minutes. NK<sub>1</sub> (L668,169) and NK<sub>2</sub> (L659,874) antagonists were prepared as 0.15 mM solutions in distilled water with 0.3% dimethylformamide, and the NK<sub>1</sub> antagonist GR82334 was a 1 mM aqueous solution; all were pH 4.5–5.0. Metabotropic glutamate (mGlu) antagonists (L- and D-2-amino-3-phosphopropionate, L- and D-AP3) and (RS)-4-carboxy-3-hydroxyphenylglycine (CHPG) were 10 mM aqueous solutions pH 8.0–8.5. PKC inhibitors chelerythrine (1 mM aqueous solution) and GF109203X (0.2 mM aqueous solution with 0.2% dimethylformamide) were pH 4.0.

All neurons were located in dorsal horn laminae IV/V, had excitatory receptive fields on the ipsilateral hindlimb (excluding glabrous skin), and were multireceptive (i.e., displayed responses to both noxious and innocuous stimuli). Neurons were initially found by their responsiveness to widespread manual brushing of the ipsilateral hindlimb. Cutaneous receptive fields of the

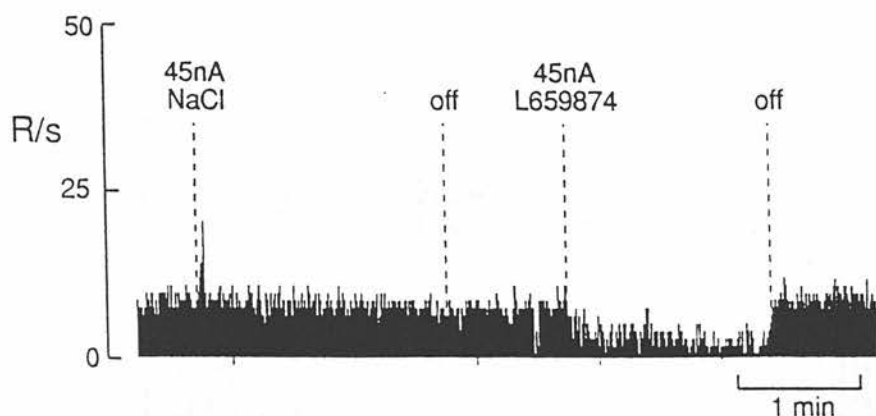


Fig. 1. Typical inhibitory effects of the NK<sub>2</sub> antagonist L659,874 on mustard oil-evoked activity of laminae IV/V dorsal horn neurons. Ionophoretic application of NaCl had no effect, whereas L659,874 consistently caused greater than 50% inhibition of evoked activity.

neurons were first localized by responses to manual brush, then the more restricted pinch and noxious heat fields were identified. The mustard oil (5–20% in paraffin oil) was applied topically with a paintbrush to the receptive fields.

## BIOCHEMISTRY

To examine the subcellular translocation of protein kinase C (PKC, a facet of its activation in response to external stimuli), we performed the following [<sup>3</sup>H]PDBu binding assay in spinal cord tissue ipsilateral/contralateral to mustard oil stimulation.

Male Wistar rats (273–330 g) were briefly anaesthetized with halothane, and mustard oil (7–20%) was applied three times to the left hindfoot and leg over a 15-minute period. This treated leg was bandaged to avoid cross-contamination and after one hour the rats were killed and the spinal cords removed and hemisected.

Each side of the spinal cord was separately homogenized in 0.4 ml of ice-cold buffer containing 20 mM Tris-HCl at pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA and 1 mM phenylmethylsulphonylfluoride with 0.01% leupeptin and 20  $\mu$ M trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64). The samples were then centrifuged at 25,000  $\times$  g for 20 minutes at 4°C. The supernatant and pellet were carefully separated and the pellet was rehomogenized in 0.3 ml Tris-BSA (50 mM Tris HCl, pH 7.4), with 4 mg/ml essential fatty acid-free bovine serum albumin. While membrane binding was carried out in Tris-BSA, cytosolic binding assays additionally contained 1 mM CaCl<sub>2</sub> and 75 mM magnesium acetate and 1.35 mg/ml sonicated

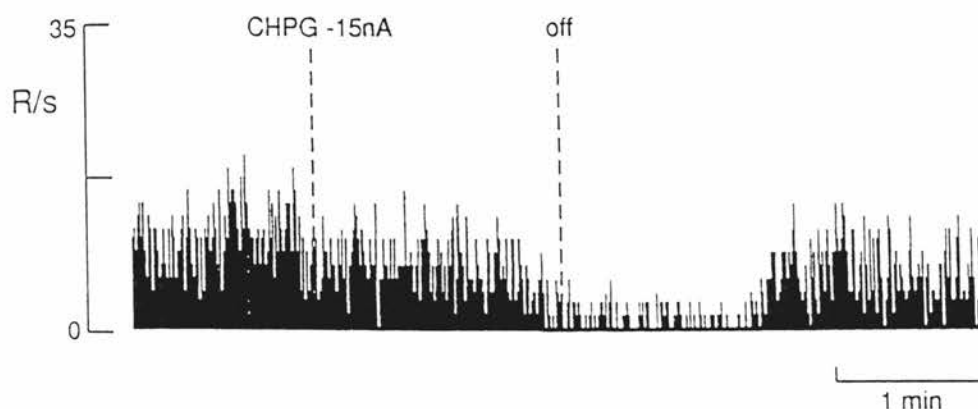


Fig. 2. An example of the inhibition of mustard oil induced firing of a dorsal horn neuron by the metabotropic glutamate receptor antagonist CHPG. In this case recovery was observed within two minutes of termination of ionophoresis of the antagonist.

phosphatidylserine, sodium salt. Assays were conducted in a total volume of 275  $\mu$ l (30 min, 37°C), with 5 nM [ $^3$ H]PDBu (approximately 0.03  $\mu$ Ci per tube) and dimethylformamide (0.5% final) or 10  $\mu$ M PDBu in dimethylformamide for total and nonspecific binding measurements, respectively. Tissue samples gave a total binding of less than 10% of total radioactivity present. Protein was precipitated at 4°C by the addition of 100  $\mu$ l of 12 mg/ml bovine gamma globulin and 300  $\mu$ l of 24% polyethyleneglycol 8000 in 50 mM Tris-HCl (pH 7.4). After 20 minutes on ice, assay tubes were centrifuged (12,000  $\times$  g, 5 min, 4°C), the supernatant aspirated, and the [ $^3$ H] radioactivity in each pellet determined after solubilization.

All laboratory chemicals were from Sigma Chemical Co., Poole, Dorset, UK, except phosphatidyl serine from Lipid Products Ltd., Nutfield, Sussex, UK. Radioligand was from Du Pont NEN, Stevenage, Herts., UK. Drugs were from Cambridge Research Biochemicals Ltd., Northwich, Cheshire, UK; Calbiochem-Novabiochem, Nottingham, Notts., UK; Tocris Neuramin, Churchill Building, Langford House, Langford, Bristol, Avon, UK; or were gifts from Merck, Sharp and Dohme and Glaxo Group Research.

## RESULTS

After application of the C-nociceptor-selective chemical algogen mustard oil, virtually all the multireceptive neurons showed a large and prolonged increase in activity ( $149 \pm 36$ -fold of background, mean  $\pm$  S.E.M.). In most cases, the steady elevated firing rate was achieved by repeated application of mustard oil 3–5 times to the same site over a period of 10–15 minutes.



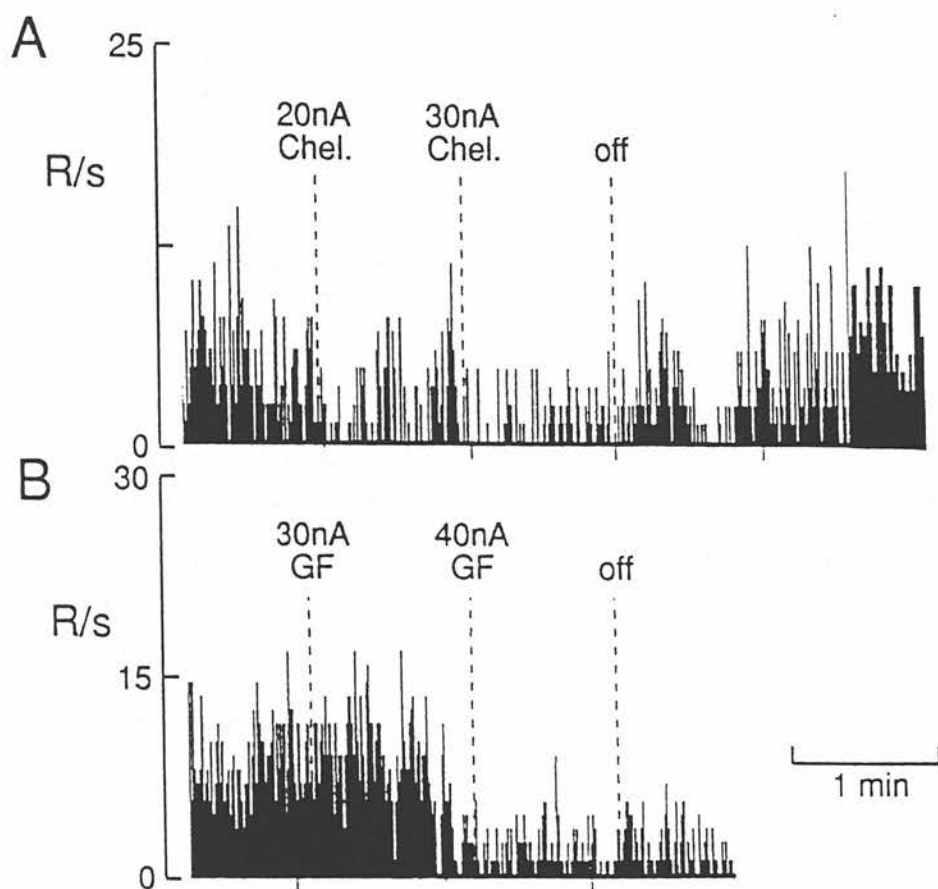


Fig. 3. The selective PKC inhibitors (A) chelerythrine and (B) GF109203X caused marked, yet recoverable inhibition of mustard oil-evoked activity.

The selective  $NK_2$  antagonist L-659,874 (10–80 nA) caused a clear and maintained inhibition of the elevated activity, to  $50 \pm 7\%$  mean  $\pm$  S.E.M., of prior control level in 23 of 24 cells (Fig. 1). Recovery was observed in 13 of 17 cases examined after terminating iontophoresis of L659,874. In contrast, the two selective  $NK_1$  antagonists L668,169 and GR82334 (20–80 nA) rarely inhibited mustard oil-evoked activity (in 1 of 11 and 3 of 11 cases, respectively). The mustard oil-evoked activity in many cells ( $n = 15$ ) was unaffected by  $NK_1$  antagonists but was inhibited by the  $NK_2$  antagonist L659,874 (Munro et al. 1993).

Selective antagonists for mGlu (L-AP3 and CHPG) and  $NK_2$  (L659,874) receptors also reliably inhibited the mustard oil-induced wind-up of neuronal activity. L-AP3 (–15 to –50 nA) and CHPG (–15 to –55 nA) inhibited the

Table I  
Mustard oil-induced translocation of specific [ $^3$ H] PDBu binding sites  
(% of total specific binding on control contralateral side)

	Cytosolic Fraction	Membrane Fraction
Control (contralateral to mustard oil)	73.7 $\pm$ 2.5	26.2 $\pm$ 2.4
Test (ipsilateral to mustard oil)	37.6 $\pm$ 5.0	40.5 $\pm$ 4.8

Note: The proportion of [ $^3$ H] PDBu binding sites in the membrane fraction was significantly greater on the test side (mean 52%) than on the control side (mean 26%)  $P < 0.05$ , Wilcoxon test). Values are the means  $\pm$  S.E.M. from nine separate experiments.

mustard oil-evoked activity (by  $68 \pm 7\%$  and  $56 \pm 9\%$  mean  $\pm$  S.E.M.) in 11 of 16 and 7 of 8 cells, respectively (Fig. 2). Recovery was sometimes but not always observed. The stereoisomer of L-AP3, D-AP3 (which has a much lower affinity for mGlu receptors; Schoepp et al. 1990b) ( $-15$  to  $-55$  nA) had no effect in 5 of 5 cells.

The selective PKC inhibitors chelerythrine and GF109203X caused marked inhibition of mustard oil-induced activity in 8 of 9 and 8 of 8 cases respectively (Fig. 3). Rapid recovery was seen in the majority of cases after removal of the PKC inhibitor. In contrast, activity evoked by continuous innocuous brushing was completely unaffected by the PKC inhibitors (data not shown,  $n = 5$ ).

In rats treated with mustard oil to one distal hindlimb, a significant translocation of specific [ $^3$ H]PDBu binding sites from the cytosolic to membrane fraction was observed in ipsilateral spinal segments L1-L5 in comparison with the contralateral (control) side (Table I).

## DISCUSSION

These results indicate that both  $NK_2$  and mGlu receptors play a crucial role in mediating the sustained excitation of lamina IV/V dorsal horn neurons elicited by cutaneous application of mustard oil. As mustard oil excites largely C-fibers (Heapy et al. 1987), this implies a role for  $NK_2$  and mGlu receptors in the transmission of nociceptive information. For tachykinins, this finding agrees with our previous studies showing that ionophoretic administration of  $NK_2$  but not  $NK_1$  receptor antagonists inhibited the responses of both rat and cat dorsal horn neurons to brief noxious thermal stimuli applied to their cutaneous receptive fields (Fleetwood-Walker et al. 1990, 1991, 1992a,b).

This evidence for a role of mGlu receptors in sustained cutaneous nociceptive inputs that lead to hyperalgesia is novel and contrasts with data from Thompson et al. (1992) who found no evidence for inhibition by L-AP3 of

ventral root potentials evoked by high-intensity stimulation of dorsal roots in an in vitro spinal cord preparation. Despite several reports of the attenuation of wind-up by NMDA receptor antagonists (Davies and Lodge 1987; Dickenson and Sullivan 1987, 1990; Thompson et al. 1990), we found no evidence for attenuation of mustard oil-evoked responses by the D-isomer of AP3 (which is a weak antagonist at the NMDA receptor [Schoepp et al. 1990b]). This may simply be a consequence of low potency of the antagonist or it may indicate a genuine mechanistic difference in the present case.

We also carried out a preliminary investigation of the intracellular mechanisms underlying the sensitization of spinal dorsal horn neurons brought about by the repeated application of mustard oil. The role of PKC in this model was clearly indicated by two separate approaches: (1) the inhibition of electrophysiological responses by ionophoretic administration of selective PKC inhibitors, and (2) translocation/activation of PKC in response to mustard oil. Although many other mechanisms may contribute to the processing of sustained nociceptive inputs and the integral sensitization of dorsal horn neurons which they bring about, this preliminary evidence clearly identifies a crucial role of PKC. Since both NK<sub>2</sub> and some mGlu receptors bring about hydrolysis of phosphoinositides, it seems likely that either or both of these receptors could well contribute to PKC activation by means of elevation of cellular diglyceride content.

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## THE ROLE OF TACHYKININS AND SOMATOSTATIN IN SPINAL NOCICEPTIVE PROCESSING

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### SUMMARY

Since not only substance P (SP) but also neurokinin A (NKA) appears to be released from fine primary afferents upon noxious cutaneous stimulation we have addressed the role of their preferential targets (NK<sub>1</sub> and NK<sub>2</sub> receptors respectively) in mediating nociceptive responses of spinal dorsal horn neurons. By making extracellular recordings of neurons in anaesthetised rats (and cats) whilst applying drugs locally by iontophoresis it was shown that NK<sub>1</sub> agonists facilitate and NK<sub>2</sub> antagonists prevent responses to brief thermal noxious stimuli in a highly selective manner. NK<sub>1</sub> receptor drugs had no marked effect, although NK<sub>1</sub> agonists could readily excite laminae IV/V but not lamina I neurons. NK<sub>2</sub> agonists excited both deep and superficial cells, apparently producing a selective desensitising block of thermal nociception in lamina I.

NK<sub>1</sub> and NK<sub>2</sub> receptor drugs were also assessed in two models of sustained C-afferent input associated with peripheral inflammation, hyperalgesia and increased responsiveness of dorsal horn. Neuronal activity wind-up over tens of minutes by cutaneous application of mustard oil was inhibited by NK<sub>2</sub> but not NK<sub>1</sub> antagonists. Expression of mRNA for preprodynorphin elicited over 6 hours by intraplantar injection of  $\lambda$ -carrageenan was similarly affected.

Experiments with another peptide released from fine nociceptive afferents, somatostatin (SS) indicated that just like NK<sub>2</sub> receptors, the SS<sub>2</sub> subtype of SS receptor may well be an essential component in mediation of brief thermal nociceptive inputs.

The interrelations between the roles played by NK<sub>1</sub>, SS<sub>2</sub> and NK<sub>2</sub> receptors (and indeed receptors for other factors co-released from nociceptive afferents) will be important to elucidate.

### INTRODUCTION

For a number of years there has been strong interest in the possibility that substance P (SP) is a transmitter mediating the input from fine primary afferent C fibres to dorsal horn neurons. In support of this hypothesis there is a body of early work describing prominent excitatory effects of SP on dorsal horn neurons, especially those responding to nociceptive

inputs (see [1] for review). More recent work with *in vitro* preparations describes facilitation by SP of responses to excitatory amino acids [2]. In behavioural experiments intrathecally-applied SP and NK<sub>1</sub> receptor agonists elicit localised biting/scratching response, perhaps consistent with irritation or pain [3] while some but not all NK<sub>1</sub> antagonists produce behavioural analgesia [4,5]. However, when the release of tachykinins into dorsal horn has been examined by antibody microprobe or push-pull cannula techniques, it is clear that SP is released by noxious levels of mechanical stimulation but surprisingly not by noxious thermal stimulation at temperatures which selectively activate polymodal C-afferents [6,7]. Higher skin temperatures however which are likely to cause inflammatory damage, do release SP [8]. In contrast, both noxious pinch and heat cause release of neurokinin A (NKA) which originates in dorsal horn very largely from primary afferents [9] where it is generated by alternative splicing of the mRNA for the SP precursor [10].

At the level of sensory responses of single neurons or nociceptive reflexes there has been relatively little decisive data implicating tachykinins as mediators of nociception; due in large part to the low specificity and potency of the early antagonists. Although SP was reported [11] to excite only those neurons that received nociceptive inputs, this has not been observed by other workers [12]. Only recently with the advent of non-peptide NK<sub>1</sub> antagonists has solid evidence been presented for an actual role of NK<sub>1</sub> receptors in mediating responses of single neurons to high intensity afferent stimulation or peripheral noxious stimuli [13,14]. More extensive studies are needed because these new agents have been found to exert prominent and very relevant effects on Ca<sup>2+</sup> channels [15]. Facilitation of a flexor reflex elicited either by SP or by conditioning stimulation of sural nerve was prevented by an improved peptide antagonist, Spantide II (a more potent blocker of NK<sub>1</sub> than NK<sub>2</sub> or NK<sub>3</sub> receptors) [16].

Our earlier studies, carried out on single neurons of anaesthetised cats, pointed clearly to a role of NK<sub>2</sub> rather than NK<sub>1</sub> receptors in mediating brief responses to noxious thermal stimuli [17]. NKA and other NK<sub>2</sub> agonists specifically facilitated such responses, whereas the early NK<sub>2</sub> antagonists [D-Pro<sup>4</sup>,Lys<sup>6</sup>,D-Trp<sup>7,9,10</sup>,Phe<sup>11</sup>]SP<sub>1-11</sub> and [D-Tyr<sup>4</sup>,D-Trp<sup>7,9</sup>,Nle<sup>11</sup>]SP<sub>1-11</sub> abrogated thermal nociceptive responses, reversed the effect of NKA on these responses and demonstrated behavioural analgesia in rat tail flick and hot plate



tests. Interestingly, selective NK<sub>1</sub> agonists had no effect on dorsal horn neurons in those studies whereas NK<sub>2</sub> agonists merely inhibited nonnociceptive responses, without modifying thermal or mechanical nociceptive responses; despite clear evidence [6] that noxious pinch releases SP into dorsal horn.

The experiments described here were carried out to further investigate the relative importance of NK<sub>1</sub> and NK<sub>2</sub> receptors in spinal nociceptive processing. There is evidence that ligand binding sites and mRNA corresponding to both NK<sub>1</sub> and to a lesser extent NK<sub>2</sub> receptors are present in superficial dorsal horn [18-20].

## METHODS

Experiments were carried out in chloralose/urethane-anaesthetised rats examining the responses of single dorsal horn neurons during localised iontophoresis of NK receptor antagonists and employing a range of sensory stimuli. Some experiments, particularly comparing the effects of tachykinins and somatostatin (SS: shown to be released into dorsal horn by noxious, but not damaging levels of thermal stimuli [6,7]) were carried out in cats. The detailed methods, and sources and specificity of the relevant pharmacological agents are described in the publications indicated.

## RESULTS

In the first series of experiments, novel and highly selective NK<sub>1</sub> and NK<sub>2</sub> antagonists were tested on the nociceptive/non-nociceptive responses of rat laminae IV/V neurons [21,22]. In 17 out of 19 neurons tested with the NK<sub>1</sub> antagonist L-659874 there was clear and selective inhibition of thermal (but not mechanical) nociceptive responses. In contrast, the NK<sub>2</sub> antagonists L-668169 and GR 82334 were without effect on nociception in 6 out of 8 and 4 out of 6 cases respectively, yet caused facilitation of non-nociceptive responses in over 75% of the cells. It seems clear that not only in cat but also in rat, NK<sub>2</sub> receptors play an important role in thermal (if not mechanical) nociception.

One possible reason for the lack of forthcoming evidence for a role of NK<sub>1</sub> receptors is that they are not present at a location accessible to the drugs as applied. To address this issue we carried out experiments with agonists. Drugs were applied iontophoretically close to laminae IV/V neurons and as a comparison, lamina I neurons [22,23]. The ascending cells of lamina I were of interest for two reasons: (i) they are reported to show a greater predominance of nociceptive cells and (ii) they are reported to receive direct synaptic contacts from fine primary afferents (including some SP reactive terminals). In laminae IV/V, selective NK<sub>1</sub> and NK<sub>2</sub> agonists produced clear excitatory effects of a similar order of magnitude which gradually declined despite continued iontophoresis.

In lamina I, NK<sub>2</sub> agonists reliably produced a marked yet transient excitation and the declining phase of this (desensitisation?) coincided remarkably with a selective diminution of responses to noxious heat. NK<sub>1</sub> agonists were without detectable effect in lamina I cells (Table 1).

Agonist	Fraction of cells excited (mean fold increase in firing rate)	
	Lamina I	Laminae IV/V
[Ac-Arg-Sar <sup>1</sup> -Met(Orn) <sup>2</sup> ]-SP <sub>1-27</sub> (NK <sub>2</sub> )	-	6/6 (x5.4 fold)
[Met(Orn) <sup>1</sup> ]-SP (NK <sub>1</sub> )	2/11 (<0.6 fold)	4/4 (x3.9 fold)
NKA (NK <sub>2</sub> )	9/11 (x11.2 fold)	6/8 (x3.5 fold)
GR 64340 (NK <sub>2</sub> )	7/9 (x7.8 fold)	5/5 (x4.0 fold)

Experiments were carried out in anaesthetised rats and drugs were applied by microiontophoresis (10-80 nA for 3-12 min) from side barrels of the multibarrelled extracellular recording electrode.

Table 1. Effects of NK receptor agonists on spontaneous electrical activity of multireceptive neurons in lamina I and laminae IV/V.

Since the sensory models investigated relied on very brief (10 sec) cutaneous stimuli, we carried out further experiments utilising models of sustained inflammatory nociceptive input to ascertain whether these in contrast would provide evidence for a role of NK<sub>1</sub> over NK<sub>2</sub> receptors. Two models were selected: (i) electrical recording of cells during topical application of mustard oil (a C-fibre selective chemical irritant) to the cutaneous receptive field, and (ii) measurement of the expression of preprodynorphin (PPD) mRNA in dorsal horn neurons (using in situ hybridisation histochemistry) following localised inflammation induced by intraplantar injection of  $\lambda$ -carrageenan. These models provide sustained C-afferent input for relatively prolonged periods and covered the experimental time domains of tens of minutes and six hours respectively. NK receptor antagonists were administered locally to the spinal dorsal horn by iontophoresis in each case (Table 2). In the mustard oil model, several NK<sub>2</sub> but not NK<sub>1</sub> antagonists again prevented mustard oil-induced excitation of spontaneous neuronal activity [24,25]. In the carrageenan model, the same profile was apparent [26,27] with no discernible evidence for a role of NK<sub>1</sub> receptors. It is possible that more profound or qualitatively different inflammatory stimuli would reveal a contribution from NK<sub>1</sub> receptors, but (at least in the present experimental conditions) it appears that NK<sub>2</sub> receptors have a more prominent role in mediating nociception. Thus, there is evidence mainly from release studies to suggest that NK<sub>2</sub> receptors may play a greater role in nociception elicited by prolonged mechanical stimuli, noxious cold or formalin [6,7,28,29].

Since release experiments [6,7] have shown that in contrast to SP, SS (as well as NKA [9]) is released into dorsal horn by noxious heat, we compared the effects of agonists and antagonists for neurokinin [17,21,22] and somatostatin [30] receptors on brief sensory responses of laminae IV/V neurons (Table 3). Whilst SS-14 had broad antinociceptive effects on responses

Antagonist	Proportion of neurons inhibited	Mean percentage inhibition in affected cells (± s.e. mean)
L 668169 (NK <sub>1</sub> )	1/11	-
GR 82334 (NK <sub>1</sub> )	3/11	(31 ± 14)
L 659874 (NK <sub>1</sub> )	23/24	50 ± 7
MEN 10376 (NK <sub>1</sub> )	4/4	38 ± 6
R 396 (NK <sub>2</sub> )	3/3	44 ± 10

Electrical activity of multi-receptive laminae IV/V neurons was recorded (as in Table 1) during topical application of mustard oil (5-20% in paraffin oil): a C-fibre selective chemical irritant [24,25]. One to five repeated applications over 10-15 min caused marked increases in firing rate (1.38 ± 0.33 fold of background, mean ± s.e. mean) which were sustained for more than 20 min and allowed testing of NK antagonists by iontophoresis (10-80 nA for 1-6 min).

a) Mustard oil-induced electrical activity:

Antagonist	Number of PPD mRNA expressing laminae I cells (% of contralateral controls: mean ± s.e. mean)	PPD mRNA silver grain density in expressing cells (% of contralateral controls: mean ± s.e. mean)
L 668169 (NK <sub>1</sub> )	98 ± 7	94 ± 7
GR 82334 (NK <sub>1</sub> )	103 ± 8	113 ± 8
L 659874 (NK <sub>1</sub> )	68 ± 5	46 ± 6

The expression of preprodynorphin mRNA in lamina I neurons of anaesthetised rats subjected to bilateral intraplantar injection of the inflammatory agent λ-carrageenan (150µl of 1.4% in saline; n=12) was measured by *in situ* hybridisation histochemistry [26,27]. Neurokinin receptor antagonists were continuously iontophoretically administered into the superficial dorsal horn of spinal segments shown to display corresponding receptive field areas. Drugs were ejected (50nA) for 1 hour prior to and then throughout the 6 hours of carrageenan stimulation. Drug effects were observed over about 700µm centred on the ejection site: the data shown are for the 200µm in closest proximity to the electrode.

b) Carrageenan-induced PPD mRNA expression

Table 2. Effects of NK receptor antagonists on the responses of dorsal horn neurons in models of sustained inflammatory nociception.

Drug (Predominant target)	Predominant effects on sensory responses (proportion of cells so influenced)		
	Noxious heat	Noxious pinch	Innocuous brush
a) Agonists			
SS-14 (SS <sub>1</sub> > SS <sub>2</sub> )	2(9/11)	2(5/5)	0(0/12)
SS-28 (SS <sub>1</sub> > SS <sub>2</sub> )	7(5/6)	0(5/5)	0(6/6)
[D-Trp <sup>6</sup> ,D-Cys <sup>14</sup> ]SS-14 (SS <sub>2</sub> > SS <sub>1</sub> )	7(5/6)	0(5/6)	0(5/6)
[Met-Ome <sup>1</sup> ]SP (NK <sub>1</sub> > NK <sub>2</sub> )	0(13/16)	0(6/9)	2(12/16)
NKA (NK <sub>2</sub> > NK <sub>1</sub> )	7(17/18)	0(11/14)	0(16/18)
b) Antagonists			
cyclo-[7-aminoheptanoyl, Phe,D-Trp,Lys,O-benzyl-Thr] (SS <sub>1</sub> /SS <sub>2</sub> )	2(7/10)	0(8/10)	0(10/11)
[D-Arg <sup>1</sup> ,D-Trp <sup>13</sup> ,Leu <sup>11</sup> ]SP (NK <sub>1</sub> > NK <sub>2</sub> )	0(10/12)	0(9/10)	0(10/12)
[D-Pro <sup>1</sup> ,Lys <sup>5</sup> ,D-Trp <sup>13</sup> ,Phe <sup>11</sup> ]SP <sub>L11</sub> (NK <sub>2</sub> > NK <sub>1</sub> )	2(11/13)	0(8/9)	0(11/13)

Experiments were carried out as described previously [17,30] on anaesthetised cats, applying drugs by microiontophoresis from a multibarrelled electrode located in lamina II immediately dorsal to the recording electrode. Since the required diffusion distances in the cat are much greater than in the rat, iontophoresis was at higher currents (350-400 nA for 6-15 min), which in ejection of 1M NaCl were shown to have no discernible effect alone on neuronal activity.

Table 3. Effects of somatostatin and neurokinin receptor agonists and antagonists on sensory responses of multi-receptive laminae IV/V neurons.

to both thermal and mechanical noxious stimuli (just as in rat [30]). SS-28 and [D-Trp<sup>6</sup>, D-Cys<sup>14</sup>]SS-14 selectively facilitated solely thermal nociceptive responses. This is analogous to the effect of the NK<sub>1</sub> agonist NKA and the parallel between effects of NKA and SS-28 is further indicated by the identical effects of their antagonists ([D-Pro<sup>1</sup>,Lys<sup>5</sup>,D-Trp<sup>13</sup>,Phe<sup>11</sup>]SP<sub>L11</sub> and cyclo-[7-aminoheptanoyl,Phe,D-Trp,Lys-O-benzyl-Thr] respectively) in selective abrogation of thermal nociception. As in the other experimental models described above, there was little evidence for a significant role of NK<sub>1</sub> receptors.

DISCUSSION

The present results provide strong support for a functionally-important role of dorsal horn NK<sub>2</sub> receptors in thermal nociception. Although present at much lower levels than NK<sub>1</sub> receptors, both ligand binding sites and mRNA for NK<sub>2</sub> receptors are present in spinal cord [18,20]. In support of our contention, the flexor reflex facilitation elicited by NKA or by conditioning stimulation of the gastrocnemius nerve is prevented by selective NK<sub>2</sub> antagonists [31]. Furthermore, the excitation of dorsal horn neurons brought about by capsaicin applied to the dorsal roots of an *in vitro* spinal cord/dorsal root preparation is similarly prevented by NK<sub>2</sub> and not NK<sub>1</sub> antagonists [32].

NK<sub>1</sub> receptors may be more important in nociception which may rely more on different classes of fine afferent inputs, for example high threshold mechanoreceptive afferents in prolonged and severe pinch-mediated responses. Both noxious cold [7] and profoundly damaging inflammatory stimuli such as intraplantar formalin [29] or ultraviolet radiation-induced burning [33] may invoke a greater NK<sub>1</sub> contribution.

It is clear that the two main processing variants from preprosomatostatin (SS-14 and SS-28) exert quite different effects on somatosensory processing. Both are present in dorsal horn [34], show selectivity for the two subtypes of SS receptor (SS<sub>1</sub> and SS<sub>2</sub>, respectively [35]) and have been shown in other CNS regions to exert quite distinct electrophysiological effects [36]. It appears that just like NKA, SS-28 may act as a transmitter of thermal nociceptive afferents. Whilst the antagonist experiments show each is necessary for maintained mediation of inputs, the full range of consequences of a thermal nociceptive input may not be felt by dorsal horn neurons unless they receive combined signals from NKA, SS-28 and possibly other co-released factors.

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## Rapid Communication

# Evidence for a Role of Metabotropic Glutamate Receptors in Sustained Nociceptive Inputs to Rat Dorsal Horn Neurons

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**Summary**—Several antagonists at metabotropic glutamate (mGlu) receptors, when applied ionophoretically, inhibited the excitation of single dorsal horn neurons elicited by cutaneous administration of the C fibre-selective algogen, mustard oil. The selectivity and stereospecificity of AP3 isomers at mGlu, compared to NMDA receptors was confirmed on responses to agonists and matched by their effects on mustard oil-evoked activity.

**Keywords**—Metabotropic glutamate receptors, dorsal horn neurons, spinal cord, nociception.

Both primary afferent neurons and local neurons of the spinal dorsal horn contain excitatory amino acids (EAA), the release of which can be elicited by noxious cutaneous stimuli or low- as well as high-intensity electrical stimulation of peripheral nerves (see refs in: Dougherty *et al.*, 1992). A number of studies have implicated NMDA receptors in nociceptive inputs from presumed A $\delta$  and C fibres, whereas AMPA-type, non-NMDA receptors may be important both there and in inputs from large myelinated fibres (Dougherty *et al.*, 1992). It is now well-established that NMDA receptors play a crucial role in the central neuronal sensitization produced by repetitive C-fibre stimulation ('wind-up') or by sustained noxious stimuli (Davies and Lodge, 1987; Haley *et al.*, 1990). Relatively little attention has been paid to the role of metabotropic glutamate (mGlu) receptors in synaptic responses in dorsal horn, although the mGlu receptor agonist (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylic acid (ACPD) potentiates metabotropic responses to AMPA and to NMDA in dispersed dorsal horn neurons (Bleakman *et al.*, 1992; Cerne and Randic, 1992).

The present experiments were carried out to assess any role of mGlu receptors in sustained activation of dorsal horn neurons by the C nociceptor-selective chemical algogen, mustard oil (allyl isothiocyanate) which causes a long-lasting sensitisation of neurons to subsequent

inputs (see refs in: Munro *et al.*, 1993). Experiments were carried out as described previously (Munro *et al.*, 1993) in adult male Wistar rats, anaesthetized with  $\alpha$ -chloralose (60 mg kg<sup>-1</sup>) and urethane (1.2 g kg<sup>-1</sup>). Extracellular recordings were made from multireceptive neurons in dorsal horn laminae III–V of segments L<sub>1</sub>–L<sub>4</sub> using the central barrel of 7-barrelled glass micro-electrodes. The remaining barrels contained 1 M NaCl for automatic current balancing (Neurophore Ionophoresis System), 2% Pontamine Sky Blue in 0.5 M sodium acetate for histological marking of recording sites and combinations of the following drugs for ionophoresis: L-1-amino-3-phosphopropanoic acid (L-AP3); D-1-amino-3-phosphopropanoic acid (D-AP3); (RS)-4-carboxy-3-hydroxyphenylglycine (CHPG); (RS)- $\alpha$ -Methyl-4-carboxyphenyl-glycine ( $\alpha$ MeCPG); (1S,3R)-1-aminocyclopentane-1,2-dicarboxylic acid (ACPD); 1-aminocyclobutane-*cis*-1,3-dicarboxylic acid (ACBD); (Tocris Neuramin); all 10 mM solutions in distilled water (adjusted pH 8.0–8.5 with NaOH).

Neuronal receptive fields were initially located by brushing of the ipsilateral hindlimb and then further examined with noxious heat (48°C, 10 sec). All neurons tested displayed a low basal firing rate of 0–1 Hz. The mustard oil experiments were carried out on a total of 28 multireceptive cells which responded to both brush and noxious heat. Repeated applications of mustard oil (7.5% in paraffin oil) were made to an area of about 3 cm<sup>2</sup> covering the receptive field, and after 2–5 applications separated by 5 min intervals, a steady elevated

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firing rate (5–13 Hz) was maintained. Antagonists were then tested by iontophoresis at currents of (–)15 to (–)55 nA. In agonist experiments, they were usually applied alone at (–)10 to (–)30 nA until a response occurred (within 1 min) and then, especially in the case of ACBD, currents were decreased to low levels in order to maintain a steady firing rate of around 5–11 Hz before antagonists were tested. The inhibitory effects of antagonists were calculated as the mean evoked activity through the 20 sec period encompassing greatest inhibition compared to the mean evoked activity in the 10 sec period immediately prior to antagonist administration.

Mustard oil-evoked activity was markedly inhibited (by  $68 \pm 7\%$ , mean  $\pm$  SEM) in 11 out of 16 cells by L-AP3 [(–)15 to (–)50 nA] which acts as a selective antagonist at a number of mGlu receptor subtypes compared to the ionotropic EAA receptors (Schoepp and Conn, 1993). In some, but not all cases, recovery was observed. In contrast, its stereoisomer D-AP3 [(–)15 to (–)55 nA], which displays an inverse selectivity for NMDA over mGlu receptors, had no effect in 5 out of 5 cells which had all shown inhibition by L-AP3 [Fig. 1(A)]. The racemic mixtures of two other mGlu receptor antagonists CHPG and  $\alpha$ MeCPG [(–)15 to (–)55 nA] caused clear inhibition in 7 out of 8 cells (by  $56 \pm 9\%$ , mean  $\pm$  SEM) and 1 out of 4 cells respectively [Fig. 1(A, B)]. In five of the cells tested with CHPG, L-AP3 was also examined and caused inhibition in each case, whilst in three of these examples, D-AP3 was also tested and had no effect.  $\alpha$ MeCPG was not tested in conjunction with other antagonists. In two cases with  $\alpha$ MeCPG, modest excitation (by no more than 20%) was observed. This may reflect the properties of the (R)-isomer in the racemic mixture, influencing other receptors in the preparation or a genuine characteristic of the mGlu receptor subtype involved here. (RS) $\alpha$ MeCPG has been demonstrated to inhibit ACPD-induced ventral root depolarization *in vitro* and the responses of ventrobasal thalamic neurons to noxious (52°C) heat (Eaton *et al.*, 1993). We did not reach the iontophoretic currents required in the latter experiments (in the order of 150 nA) so the relative inactivity of  $\alpha$ MeCPG here may simply be due to a failure to reach adequate concentrations.

In order to confirm the selectivity of some of the antagonists under the present conditions they were also tested on responses to the mGlu receptor agonist, ACPD and the NMDA receptor agonist, ACBD. These were different neurons from those tested with mustard oil so as to avoid any possibility of agonist administration modifying mustard oil responses. Fourteen out of 37 cells tested responded to ACPD and 16 out of 23 responded to ACBD. Of the neurons responding to ACPD or ACBD, 9 out of 12 and 7 out of 12 tested respectively, had also previously shown vigorous excitatory responses to noxious heat. In 20 cells tested with both agonists, six gave clear responses to both, three responded selectively to ACPD, nine responded selectively to ACBD and two were unaffected. In each

category there were examples of cells which either did or did not respond overtly to noxious heat. The effects of agonists on subsequent sensory responses were not examined in these experiments. In cells considered as failing to respond to agonists, ACPD was tested at currents to (–)90 nA and ACBD at currents up to (–)80 nA. In several cells agonist responses were tested comparatively with L-AP3 and D-AP3. In 4 out of 6 neurons activated by ACPD at (–)7 to (–)25 nA, L-AP3 [(–)15 to (–)30 nA] caused marked inhibition of activity by  $67 \pm 12\%$  (mean  $\pm$  SEM) whereas in all cases D-AP3 had either no effect or caused slight excitation [Fig. 1(C)]. In contrast, D-AP3 [(–)15 to (–)30 nA] inhibited the activity elicited by ACBD [(–)1 to (–)6 nA] in 4 out of 6 neurons by  $58 \pm 17\%$  (mean  $\pm$  SEM), whereas L-AP3 had no effect in any of them [Fig. 1(D)]. In one example we were able to record the expected selectivity of AP3 isomers on activity evoked by ACPD and then, after recovery, the inverse selectivity on activity evoked by ACBD. In 3 out of 3 further cells, L-AP3 [(–)15 to (–)30 nA] inhibited ACPD-evoked activity by  $76 \pm 11\%$  (means  $\pm$  SEM). CHPG [(–) 30 to (–) 50 nA] caused 46% and 97% inhibition in two of these cells.  $\alpha$ MeCPG was not tested.

These results demonstrate that a number of mGlu receptor antagonists inhibit the sustained activation of dorsal horn neurons elicited by repeated application of a selective chemical stimulant of C-nociceptors. Stereoselectivity of inhibition was observed in the effectiveness of L-AP3 but not D-AP3. Pharmacological selectivity of L-AP3 and D-AP3 for mGlu and NMDA receptor responses respectively was demonstrated in the majority of cells under the present experimental conditions using exogenous selective agonists.

Examples where no effect was seen may have resulted from inadequate access or conceivably from contributions of different subtypes of these receptor classes. Indeed the cells sampled for the agonist experiments may represent in part a different population from those in the mustard oil experiments. Notably, however, we never observed blockade of ACPD responses by D-AP3 or of ACBD responses by L-AP3.

Although D-AP3 had no effect on mustard oil-evoked activation in these experiments, it is a weak NMDA receptor antagonist and we have not attempted to investigate effects of more potent and selective NMDA receptor antagonists. There is good evidence that in other models of sustained C-afferent activation of dorsal horn neurons (Davies and Lodge, 1987; Haley *et al.*, 1990) NMDA receptors play an important role. The contribution of NMDA receptors to mustard oil-induced activation here is thus not clear and in the present study we have sought only to elucidate whether mGlu receptors may play a role. The present evidence for involvement of an L-AP3-sensitive mGlu receptor contrasts with observations on long duration ventral root potentials evoked by dorsal root stimulation in neonatal rat spinal cord *in vitro*, which are sensitive to 2-amino-5-phosphonopentanoic acid but not L-AP3 (Thompson *et al.*, 1992). It seems likely that different



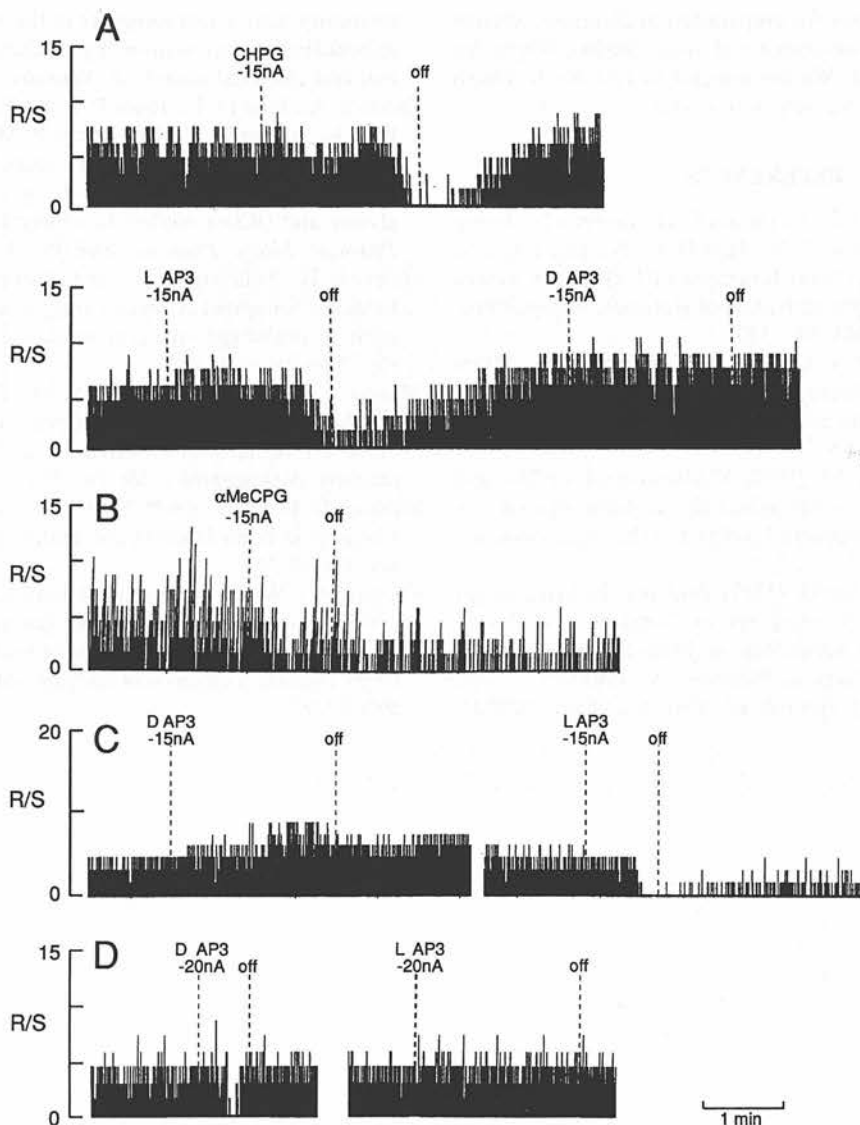


Fig. 1. Inhibition of mustard oil-evoked activity of dorsal horn neurons by metabotropic glutamate (mGlu) receptor antagonists. Action potentials of recorded cells were clearly discriminated from other field potentials and the ongoing firing frequency in Hz (Responses/sec, R/S) was displayed continuously. Basal activity in all records was less than 1 Hz. Records (A) and (B) show activity evoked by prior application of 7.5% mustard oil on 2–5 occasions at 5 min intervals to the cutaneous receptive field of the neurons. (C) Shows activity evoked by ionophoresis of the mGlu receptor agonist ACPD (1–2 nA for 7 min prior to and during the test). (D) Shows activity evoked by ionophoresis of the NMDA receptor agonist ACBD (1 nA for 10 min prior to and during the test). In each record, antagonists were tested at the currents and durations indicated. The inhibitory effect of L-AP3, CHPG and  $\alpha$ MeCPG (but not D-AP3) seen on mustard oil-evoked activity (A, B) was paralleled by the inhibitory effect of L-AP3 (but not D-AP3) on ACPD-evoked, but not ACBD-evoked activity (C, D).

models of nociception and different cell populations may state the extent to which particular EAA receptors contribute to sustained nociceptive responses. Whilst prominent activation by ACPD and inhibition by L-AP3 appear to preclude some subtypes of mGlu receptors (Choepf and Conn, 1993), it is not yet possible to define the particular species of mGlu receptor involved here. Other mediators are likely to contribute additionally to mustard oil-induced activation and sensitization of dorsal horn neurons, since we have shown previously that NK-2 receptor antagonists also inhibit this neuronal activation (Munro *et al.*, 1993). There is reason to

suspect that mGlu receptors may interact functionally with ionotropic Glu receptors during sustained nociceptive responses since ACPD augments and mGlu antagonists prevent tetanus-induced long-term potentiation in hippocampal CA1 neurons (Bashir *et al.*, 1993) while ACPD augments the effects of exogenous ionotropic receptor agonists on isolated dorsal horn neurons (Bleakman *et al.*, 1992; Cerne and Randic, 1992).

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In view of previous electrophysiological evidence that NK<sub>1</sub>, NK<sub>2</sub> and metabotropic glutamate (mGlu) receptors contribute to nociceptive inputs to dorsal horn neurons (Fleetwood-Walker *et al.* 1993; Thompson *et al.* 1993; Young *et al.* 1994), we assessed whether antagonists at these receptors were effective in normal and carrageenan-inflamed models of thermal and mechanical nociception. Lister-hooded rats (30-70g) were intrathecally (i.t.) injected (10µl) with vehicle (saline or 0.3% dimethylsulphoxide in saline) or drug, following measurement of baseline paw withdrawal latencies (PWL) for both noxious mechanical and thermal stimuli, applied to the dorsal hairy foot. A 100µl ipsilateral sub-cutaneous injection of 2% lambda-carrageenan was given under brief halothane anaesthesia. Paw withdrawal latencies measured 3 hours later showed an average decrease of approximately 50% from pre-carrageenan levels. Drug effects were then tested 1, 3, and 5 mins after i.t. injection. In the non-inflamed state, the NK<sub>2</sub> receptor antagonist L659874 (3nmol, n=6) raised thermal (but not mechanical) PWL by 83±26% above pre-drug baseline levels (P<0.01 Mann-Whitney U-test, with respect to vehicle controls). In carrageenan-treated animals, L659874 (n=14) resulted in similar changes in thermal PWL (+91±21%, P<0.01). The mGlu receptor antagonist L-1-amino-3-phospho-propanoic acid (L-AP3; 60nmol, n=14) was ineffective, whereas (S)-4-carboxy-3-hydroxyphenylglycine ((S)CHPG; a second mGlu antagonist, 48nmol, n=14) showed increased mechanical and thermal PWLs in the non-inflamed group (+49±10%, P<0.05 and +40±8%, P<0.01, respectively). With

carrageenan treatment, L-AP3 (n=14) was now found to increase PWL to both mechanical and thermal stimulation (+156±58, P<0.05 and +97±5%, P<0.01, respectively). The effects of L-AP3 were not mimicked by D-AP3. Subsequent to carrageenan inflammation, (S)CHPG (n=13) produced more profound increases in mechanical and thermal PWL (+130±31%, P<0.01 and +74±10%, P<0.05, respectively). The NK<sub>1</sub> receptor antagonist GR92334, at intrathecal doses (0.7-1 nmol), previously shown to effectively antagonize the caudally-directed biting and scratching evoked by the NK<sub>1</sub> agonist substance P methyl ester (Birch *et al.* 1991), was without effect on either mechanical or thermal PWL in untreated or carrageenan-treated animals under the present experimental conditions. Nevertheless NK<sub>1</sub> antagonists have been indicated to play a significant role in the enhanced nociceptive responses elicited by UV-light-induced inflammation or C-afferent conditioning stimulation (Thompson *et al.* 1994; Xu *et al.* 1992). These data are consistent with a role for NK<sub>1</sub> receptors in the transmission of brief thermal nociceptive inputs and for mGlu receptors in both mechanical and thermal nociceptive responses in this carrageenan model.

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# The Involvement of Metabotropic Glutamate Receptors and Their Intracellular Signalling Pathways in Sustained Nociceptive Transmission in Rat Dorsal Horn Neurons

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**Summary**—The excitatory responses of individual dorsal horn neurons to cutaneous brush, repeated application of the C-fibre-selective chemical algogen, mustard oil, or to ionophoretic (1S,3R)-ACPD [a metabotropic glutamate receptor (mGluR) agonist] were monitored by extracellular recording. We have previously shown that the responses of dorsal horn neurons to mustard oil are inhibited by several selective antagonists of mGluRs. Effects of ionophoresis of the mGluR antagonists (R,S)-CHPG and L-AP3 and a range of selective inhibitors of intracellular signalling pathways were examined on evoked responses here. The results suggest that protein kinase C, phospholipase A<sub>2</sub> and perhaps Ca<sup>2+</sup>/calmodulin kinase II play a role in mediating the sustained elevated activity of dorsal horn neurons that is incrementally elicited by repeated application of mustard oil, but probably make little contribution to sustained brush-evoked activity. Concurrence in the sensitivity of mustard oil- and (1S,3R)-ACPD-evoked activity to (R,S)-CHPG, L-AP3 and to inhibitors of intracellular signalling pathways, suggests that mGluRs are an important origin of these intracellular signals required for sustained nociception.

**Keywords**—Metabotropic glutamate receptors, nociception, spinal cord, intracellular signalling, protein kinase C, phospholipase A<sub>2</sub>.

The possible role of metabotropic glutamate receptors (mGluRs) in spinal sensory processing has been investigated relatively little. Their potential importance is highlighted by evidence for their role in gating NMDA receptor-dependent and -independent forms of the sensitization phenomenon in the hippocampus known as long-term potentiation (LTP) (Bortolotto and Collingridge, 1993; Bortolotto *et al.*, 1994) and by the facilitation by mGluR agonist [(1S,3R)-ACPD] of AMPA and NMDA responses in dorsal horn neurons (Leakman *et al.*, 1992; Cerne and Randic, 1992). We have previously provided evidence for a mediatory role of mGluRs in the sustained activation of dorsal horn neurons elicited by repeated cutaneous application of the C-fibre selective chemical algogen, mustard oil (Young *et al.*, 1994), which is known to bring about central sensitization of dorsal horn neurons to afferent inputs. The repeated cutaneous application of mustard oil elicits an increasingly powerful and sustained activation of

dorsal horn neurons (analogous to “wind-up”) until a state of persistent elevated activity is reached (Munro *et al.*, 1993). This persistent mustard oil-evoked activity was inhibited by ionophoretic application of the selective mGluR antagonists L-(but not D)-1-amino-3-phosphonopropanoic acid (L-AP3), (R,S)-4-carboxy-3-hydroxyphenylglycine (R,S-CHPG) and, more variably, by (R,S)- $\alpha$ -methyl-4-carboxyphenylglycine (R,S- $\alpha$ -MeCPG) (Young *et al.*, 1994). In behavioural analgesia experiments, intrathecally-applied (S)-CHPG, which is the enantiomer with activity at mGluRs, produces antinociceptive effects in thermal and mechanical paw withdrawal tests (Young *et al.*, 1995). In animals treated by unilateral intraplantar injection of carrageenan, these effects are amplified and an antinociceptive influence of L-AP3, but not D-AP3, is also apparent (Young *et al.*, 1995). Co-administration of AMPA and mGluR agonists is reported to bring about behavioural hyperalgesia in acute mechanical nociceptive tests (Meller *et al.*, 1993). The sensitization of dorsal horn neurons brought about by intra-articular inflammation is also inhibited by the mGluR antagonist L-AP3 (Neugebauer *et al.*, 1994).

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While these data provide compelling evidence that mGluRs play some role in spinal processing of nociceptive inputs (most likely in the sensitization of dorsal horn neurons to sustained high intensity C-fibre input), any evidence is lacking as to their intracellular mechanism of action. We have previously reported that neuronal activity evoked by repeated mustard oil application is profoundly inhibited by antagonists of protein kinase C (PKC) (Munro *et al.*, 1994a). This corresponds to the attenuation of behavioural nociceptive responses to subcutaneous formalin injection (and of their facilitation by glutamate) seen with PKC inhibitors (Coderre, 1992; Coderre and Yashpal, 1994). Since the intracellular signals leading to PKC activation are likely to also involve elevation of  $\text{Ca}^{2+}$  levels, it is possible that the  $\text{Ca}^{2+}$ -activated enzyme,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II [which has been implicated in LTP (Silva *et al.*, 1992)], may also play a role here. Additionally, evidence for the involvement of prostanoids in the nociceptive responses to formalin (Malmberg and Yaksh, 1992a,b; Chapman and Dickenson, 1992) and the facilitation of such behavioural responses by the prostanoid precursor arachidonic acid (Coderre and Yashpal, 1994), suggests that the arachidonic acid-generating enzyme, phospholipase  $\text{A}_2$  ( $\text{PLA}_2$ ) may play a role in sensitization. Further transduction mechanisms which could potentially play a role are cAMP-dependent protein kinase (PKA), since PKA activation can potentiate NMDA responses of dorsal horn neurons (Cerne *et al.*, 1993), and non-receptor tyrosine kinases, which can be activated by a number of phosphoinositide-hydrolysing, G protein-coupled receptors (Zachary *et al.*, 1991).

The present experiments utilized a range of selective inhibitors applied by iontophoresis, to assess the role of these various signal transduction pathways in neuronal responses to repeated application of mustard oil, to activation by the mGluR agonist (1*S*,3*R*)-1-aminocyclopentane-1,2-dicarboxylic acid [(1*S*,3*R*)-ACPD] and to light innocuous brushing of the cutaneous receptive field. Whilst these reagents were all chosen for their reported high degree of selectivity, it is not possible to say with certainty that their expected effects represent the actual mechanism of action in the present experiments. For this reason, pairs of reagents (with quite different structures, but with the common property of selectively blocking particular signal transduction pathways) were tested in each case.

## METHODS

Experiments were carried out on 43 male Wistar rats (240–420 g). Following an initial halothane anaesthesia, animals were given intravenous  $\alpha$ -chloralose (60 mg  $\text{kg}^{-1}$ ) and urethane (1.2 g  $\text{kg}^{-1}$ ). Supplementary doses of  $\alpha$ -chloralose were given when required. Core body temperature was maintained at 37–38°C by means of a thermostatically controlled heat blanket. A light flow of  $\text{O}_2$  (0.1 l  $\text{min}^{-1}$ ) was passed over the end of a tracheal

cannula to enrich the inspired air. Once the thoraco-lumbar spinal column was supported, using 3 pairs of swan-necked clamps, a laminectomy ( $\text{L}_1$ – $\text{L}_4$ ) was carried out. To provide stability, agar was injected under the most rostrally clamped vertebra, then over the whole area of the laminectomy. An agar core was removed to expose the recording region. The dura was then cut and a pool of 37°C liquid paraffin applied to the region.

Extracellular recordings were made from neurons in the deeper dorsal horn of spinal segments  $\text{L}_1$ – $\text{L}_4$ , using the central barrel of 7-barrelled glass microelectrodes. The remaining barrels contained 1 M NaCl for automatic current balancing (Neurophore Iontophoresis System). 2% Pontamine Sky Blue in 0.5 M sodium acetate for histological marking of recording sites and combinations of the following drugs for iontophoresis: (1*S*,3*R*)-ACPD (Irving *et al.*, 1990), 10 mM aqueous, pH 8.0–8.5; (*R,S*)- and (*S*)-CHPG (Watkins and Collingridge, 1994), 10 mM aqueous, pH 8.0–8.5; L-AP3 (Schoepp *et al.*, 1990), 10 mM aqueous, pH 8.0–8.5; calmidazolium (Silver *et al.*, 1986), 50  $\mu\text{M}$  in 0.5% dimethylformamide, pH 4.0–4.5; KN62 (Tokumitsu *et al.*, 1990), 50  $\mu\text{M}$  in 0.5% dimethylformamide, pH 4.0–4.5; GF109203X (Toullec *et al.*, 1991), 100  $\mu\text{M}$  in 0.5% dimethylformamide, pH 4.0–4.5; chelerythrine (Herbert *et al.*, 1990), 1 mM aqueous, pH 4.0–4.5; ONO-RS-082 (Banga *et al.*, 1986), 100  $\mu\text{M}$  in 0.5% dimethylformamide, pH 4.0–4.5; benzenesulphonamide 4 (BS4; Oinuma *et al.*, 1991), 50  $\mu\text{M}$  in 0.5% dimethylformamide, pH 4.0–4.5; KT5720 (Kase *et al.*, 1987), 50  $\mu\text{M}$  in 0.5% dimethylformamide, pH 4.0–4.5; H89 (Chijiwa *et al.*, 1990), 100  $\mu\text{M}$  in 0.5% dimethylformamide, pH 4.0–4.5; lavendustin A (Hsu *et al.*, 1991), 200  $\mu\text{M}$  in 0.2% dimethylformamide, pH 4.0–4.5; and piceatannol (Geahlen and McLaughlin, 1989), 300  $\mu\text{M}$  in 0.3% dimethylformamide, pH 4.0–4.5. (1*S*,3*R*)-ACPD, (*R,S*)-, (*S*)-CHPG and L-AP3 were from Tocris Neuramin, calmidazolium was from Sigma, piceatannol was from Boehringer Mannheim and all other compounds were from Calbiochem, except BS4 which was a gift from Mike Clark (Schering-Plough Research, Kenilworth, NJ, U.S.A.), and ONO-RS-082 which was a gift from Dr Tsuboshima (ONO Pharmaceuticals, Osaka, Japan). The signal transduction reagents were dissolved at the maximum concentration that could be achieved in an acceptable vehicle (0.5% dimethylformamide) that did not affect neuronal activity (see below). With certain reagents it was possible to use lower concentrations of the solvent in iontophoretic solutions.

Neuronal receptive fields were initially located by brushing of the ipsilateral hindlimb and then further examined with noxious heat (48°C, 10 sec). All neurons tested displayed a low basal firing rate of 0–1 Hz. The mustard oil experiments were carried out on a total of 38 multireceptive cells which responded to both brush and noxious heat. Repeated applications of mustard oil (Allyl isothiocyanate, Aldrich Chemical Company, 7.5% in paraffin oil) were made to an area of about 3  $\text{cm}^2$  covering

receptive field, and after 2–5 applications separated by 1 min intervals, a steady elevated firing rate (4–37 Hz) was maintained. The inhibitory effects of antagonists were calculated as the mean evoked activity through the 20 or 30 sec period encompassing greatest inhibition, compared to the mean evoked activity in the 20 or 30 sec period immediately prior to antagonist administration. Analysis was routinely over 20 sec, but was occasionally for the longer period of 30 sec if spurious variations occurred in the control sampling period, so that more truly representative values were obtained. The effects of antagonists, over a similar current range to that used on mustard oil-evoked activity, were also tested on responses of cells to a motorized brush applied continuously to the cutaneous receptive field and to ionophoretically-applied (1*S*,3*R*)-ACPD. The ionophoretic current ranges used were the lowest found to cause > 50% reduction of mustard oil-evoked activity without any detectable spike distortion; equivalent currents were then tested on brush- and (1*S*,3*R*)-ACPD-evoked responses. The statistical significance of drug-induced changes from control responses was assessed by the Wilcoxon test on the mean evoked raw firing rate in the sampling periods before and during drug administration. All analysed activity data are quoted as mean  $\pm$  SEM values.

## RESULTS

The majority of the neurons examined were multireceptive (i.e. responded to both innocuous brushing and noxious pinch or noxious heating to 48°C, of the cutaneous receptive field). As described previously (Munro *et al.*, 1993; Young *et al.*, 1994), the majority of these were vigorously excited by cutaneous administration of mustard oil, with several successive applications resulting in sustained elevated activity. The majority of Pontamine Sky Blue spots recovered from neuronal recording sites were within laminae III–V of the dorsal horn. Of the population of neurons recorded in the present study, approximately one-fifth (47 out of 244) were overtly excited by ionophoresis of (1*S*,3*R*)-ACPD (0–60 nA). Ionophoresis of 0.5% dimethylformamide-containing vehicle or saline at up to 80 nA, had no detectable or consistent effect on basal, mustard oil-, (1*S*,3*R*)-ACPD- or brush-evoked responses ( $n = 5$ ). For each mGluR antagonist or signal transduction reagent, whenever recovery of responses was examined, full recovery was seen in most cases by 10 sec–15 min after ionophoresis was stopped.

Figure 1 extends the previously reported observations (Young *et al.*, 1994; Munro *et al.*, 1994b) that mGluR antagonists inhibit mustard oil-evoked neuronal activation, by demonstrating that the potent mGluR antagonists (*R,S*)-CHPG and L-AP3 act selectively to inhibit mustard oil-evoked activity without affecting that evoked by innocuous brush. Mustard oil-evoked activity was significantly inhibited by (*R,S*)-CHPG [(–)15 to (–)55 nA] and L-AP3 [(–)15 to (–)50 nA] in 7 out of 8

cells ( $56 \pm 9\%$  inhibition,  $P < 0.05$ ) and 11 out of 16 cells ( $68 \pm 7\%$  inhibition,  $P < 0.05$ ), respectively (Wilcoxon test on raw data) (Fig. 1; Young *et al.*, 1994; Munro *et al.*, 1994b). In contrast, brush-evoked activity was never altered by more than 15% in 5 out of 5 cells by (*R,S*)-CHPG and 9 out of 9 cells tested with L-AP3 (not statistically significant). Furthermore, 12 out of 12 cells tested with (*S*)-CHPG [(–)15 to (–)55 nA] also showed negligible effects (<15% inhibition on brush-evoked activity (not statistically significant). Similar to their effect on mustard oil-evoked activity, (*R,S*)-CHPG and L-AP3 inhibited (1*S*,3*R*)-ACPD-driven activity in 5 out of 5 and 4 out of 6 cells, respectively (Fig. 1; Young *et al.*, 1994). The effect of (*R,S*)-CHPG on (1*S*,3*R*)-ACPD-evoked activity was a mean inhibition of  $64 \pm 6\%$  (5/5 neurons) at currents of (–)15 to (–)40 nA ( $P < 0.05$ ). When the percentage inhibition values of brush-, mustard oil- and (1*S*,3*R*)-ACPD-evoked activity caused by (*R,S*)-CHPG were compared by the non-parametric Mann–Whitney *U*-test, effects on each of the other responses were significantly greater than those on brush ( $P < 0.05$ ). As a consequence of the lasting nature of activation caused by mustard oil, it was not possible to test drug effects in a side-by-side comparison of brush-, mustard oil- and (1*S*,3*R*)-ACPD-evoked responses on individual cells. Comparisons of a drug effect on brush/mustard oil, brush/(1*S*,3*R*)-ACPD or (1*S*,3*R*)-ACPD/mustard oil were however made in a number of cases. In individual examples of each combination, both (*R,S*)-CHPG and L-AP3 caused similar inhibition (by > 50%) of mustard oil/(1*S*,3*R*)-ACPD responses, but in examples of sequential brush/mustard oil and brush/(1*S*,3*R*)-ACPD tests the antagonists inhibited brush responses by <15%, if at all, despite causing > 50% reduction in mustard oil and (1*S*,3*R*)-ACPD responses.

When a range of selective inhibitors of signal transduction pathways was tested on activity evoked by brush, mustard oil or (1*S*,3*R*)-ACPD, there was general concurrence in the effects of each inhibitor on mustard oil- and (1*S*,3*R*)-ACPD-evoked responses. These responses were significantly inhibited by ionophoresis of each of the agents tested ( $P < 0.05$ , by Wilcoxon test, in each case; Table 1). However, in the case of certain inhibitors of  $\text{Ca}^{2+}$ /calmodulin kinase II, PKC and  $\text{PLA}_2$ , but not the other agents, there appeared to be a lesser effect on brush-evoked activity than on the other responses. The  $\text{Ca}^{2+}$ /calmodulin kinase II inhibitor, calmidazolium, the PKC inhibitor GF 109203X, and the  $\text{PLA}_2$  inhibitor ONO-RS-082 had no significant effect on brush-evoked activity. The mean percentage inhibition of mustard oil- and (1*S*,3*R*)-ACPD-evoked responses by calmidazolium, ONO-RS-082 and a further  $\text{PLA}_2$  inhibitor, BS4, was consistently at least 2-fold greater than their effect on brush responses (and in the case of GF109203X, many fold more). In the case of GF109203X, the percentage inhibition of mustard oil and of (1*S*,3*R*)-ACPD responses was significantly greater

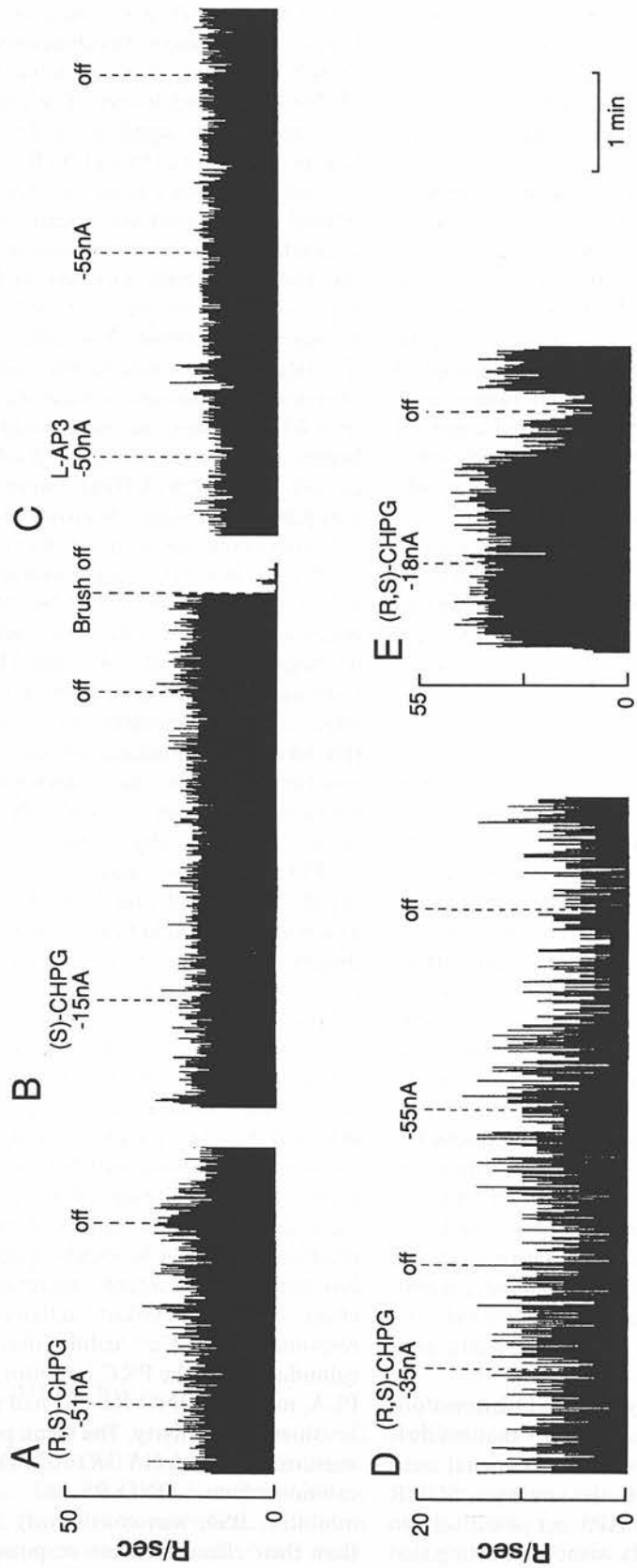


Fig. 1. Effects of ionophoretically-applied mGluR antagonists on evoked responses of dorsal horn neurons. Individual records of ongoing firing frequency are displayed as the action potentials per second (R/sec), integrated over 700 msec bins, plotted against time. (A–C) show the typical lack of any marked effect of mGluR antagonists on brush-evoked activity; (D, E) show inhibitory effects of (R,S)-CHPG on mustard oil- and (1S,3R)-ACPD-evoked activity (8 nA), respectively. Each trace is from a separate neuron and is entirely typical of the results obtained in the overall population sampled here.

than that of brush responses ( $P < 0.05$ , Mann-Whitney  $U$ -test). The effect of ONO-RS-082 on (1*S*,3*R*)-ACPD-evoked activity was also significantly greater than that on brush ( $P < 0.05$ ), but no other such "between-response" comparisons revealed differences that were significant with the present samples. Figure 2 shows examples of the effect of the  $\text{Ca}^{2+}$ /calmodulin kinase II inhibitor KN62 and of the  $\text{PLA}_2$  inhibitor BS4, illustrating concurrent inhibition of mustard oil- and (1*S*,3*R*)-ACPD-evoked activity with little or no effect on brush responses. In the overall populations, however, both KN62 and BS4 caused a relatively small, but significant, inhibition of brush as well as the other responses, with each drug showing no marked effect ( $\leq 20\%$  change) in approximately 50% of the cells (as in the examples illustrated) (Fig. 2; Table 1). In the case of the PKA inhibitors, KT5720 and H89, and the tyrosine kinase inhibitors, lavendustin A and piceatannol, each reagent tested caused a significant inhibition of all types of evoked activity (Table 1). It was not possible to make direct sequential comparisons of a drug's effects on brush, mustard oil and (1*S*,3*R*)-ACPD responses of the same cell. However, comparisons between two types of evoked response were possible in a number of cases. For example, in the case of a single neuron tested with BS4, direct comparison of effects on brush/(1*S*,3*R*)-ACPD responses

revealed selective inhibition of the latter, in accordance with the overall population of neurons investigated. Similarly, piceatannol caused inhibition of both brush and mustard oil responses when directly compared on a single cell, again typical of the overall population (Table 1). A total of 17 cells were tested sequentially with several drugs on either responses to brush (6 cells), mustard oil (6 cells) and (1*S*,3*R*)-ACPD (5 cells). In order to avoid any possible cumulative drug effects, this involved drugs considered to act on independent signal transduction pathways. Time for full recovery was always allowed between sequential tests on the same neuron (up to 15 min) and the results from these multiple tests were always typical of the overall population described in Table 1.

## DISCUSSION

The present experiments extend the evidence for a role of mGluRs in mediating the prolonged firing of dorsal horn neurons evoked by repeated application of mustard oil. Furthermore, they demonstrate modality specificity, in that neuronal activation caused by mustard oil, but not that due to continuous innocuous brushing, was inhibited by the mGluR antagonists (*R,S*)-CHPG and L-AP3 (Fig. 1). Administration of the single enantiomers

Table 1. Effects of ionophoretically-applied inhibitors of intracellular signalling pathways on the activity of dorsal horn neurons evoked by brush, mustard oil or (1*S*,3*R*)-ACPD

Principal target	Drug	Inhibition of evoked activity (% inhibition compared to mean pre-drug controls)		
		Brush	Mustard oil	(1 <i>S</i> ,3 <i>R</i> )-ACPD (0–10 nA)
Calmodulin, $\text{Ca}^{2+}$ /calmodulin kinase	Calmidazolium (30–60 nA)	16 $\pm$ 10 NS $n = 8$ (25%)	42 $\pm$ 14* $n = 7$ (71%)	68 $\pm$ 16* $n = 5$ (80%)
	KN62 (30–70 nA)	42 $\pm$ 13* $n = 12$ (50%)	51 $\pm$ 19* $n = 5$ (80%)	83 $\pm$ 9* $n = 5$ (100%)
PKC	GF109203X (20–60 nA)	3 $\pm$ 3 NS $n = 5$ (20%)	53 $\pm$ 10* $n = 9$ (89%)	86 $\pm$ 12* $n = 5$ (100%)
	Chelerythrine (10–80 nA)	—	70 $\pm$ 10* $n = 8$ (100%)	84 $\pm$ 11* $n = 7$ (100%)
$\text{PLA}_2$	ONO-RS-082 (30–65 nA)	28 $\pm$ 13 NS $n = 9$ (44%)	70 $\pm$ 11* $n = 5$ (100%)	75 $\pm$ 9* $n = 8$ (100%)
	BS4 (30–60 nA)	37 $\pm$ 12* $n = 9$ (56%)	74 $\pm$ 11* $n = 5$ (100%)	86 $\pm$ 6* $n = 7$ (100%)
PKA	KT5720 (20–60 nA)	59 $\pm$ 15* $n = 10$ (70%)	73 $\pm$ 13* $n = 7$ (86%)	87 $\pm$ 11* $n = 5$ (80%)
	H89 (30–70 nA)	45 $\pm$ 15* $n = 10$ (50%)	66 $\pm$ 10* $n = 5$ (100%)	79 $\pm$ 13* $n = 6$ (100%)
Tyrosine kinases	Lavendustin A (30–70 nA)	60 $\pm$ 12* $n = 11$ (82%)	58 $\pm$ 10* $n = 8$ (88%)	69 $\pm$ 12* $n = 7$ (100%)
	Piceatannol (7–50 nA)	48 $\pm$ 15* $n = 13$ (61%)	87 $\pm$ 7* $n = 6$ (83%)	84 $\pm$ 9* $n = 6$ (100%)

The ranges of ionophoretic currents required for the effects reported, are indicated in parentheses below each drug. In the case of (1*S*,3*R*)-ACPD, higher currents ( $\leq 60$  nA) were sometimes briefly used to initiate firing but, after the first few seconds, the elevated activity could always be maintained by  $\leq 10$  nA.

The effects of the drugs on evoked responses are indicated as the mean  $\pm$  SEM percentage inhibition compared to pre-drug control activity. In each case, the number of neurons contributing to the data is shown below and in parentheses, the proportion (percentage) of those neurons which individually gave a clear response of  $> 20\%$  inhibition. The statistical significance of drug-induced changes in firing rate was assessed using the Wilcoxon test on mean raw firing rate data in action potentials per second (R/sec); comparing the 20 or 30 sec period immediately prior to drug administration with the 20 or 30 sec period centred on the greatest inhibitory effect of drug (\*represents  $P < 0.05$ ; NS represents not significantly different,  $P > 0.05$ ).

The data for GF109203X and chelerythrine on brush- and mustard oil-evoked activity is taken from Munro *et al.* (1994a).



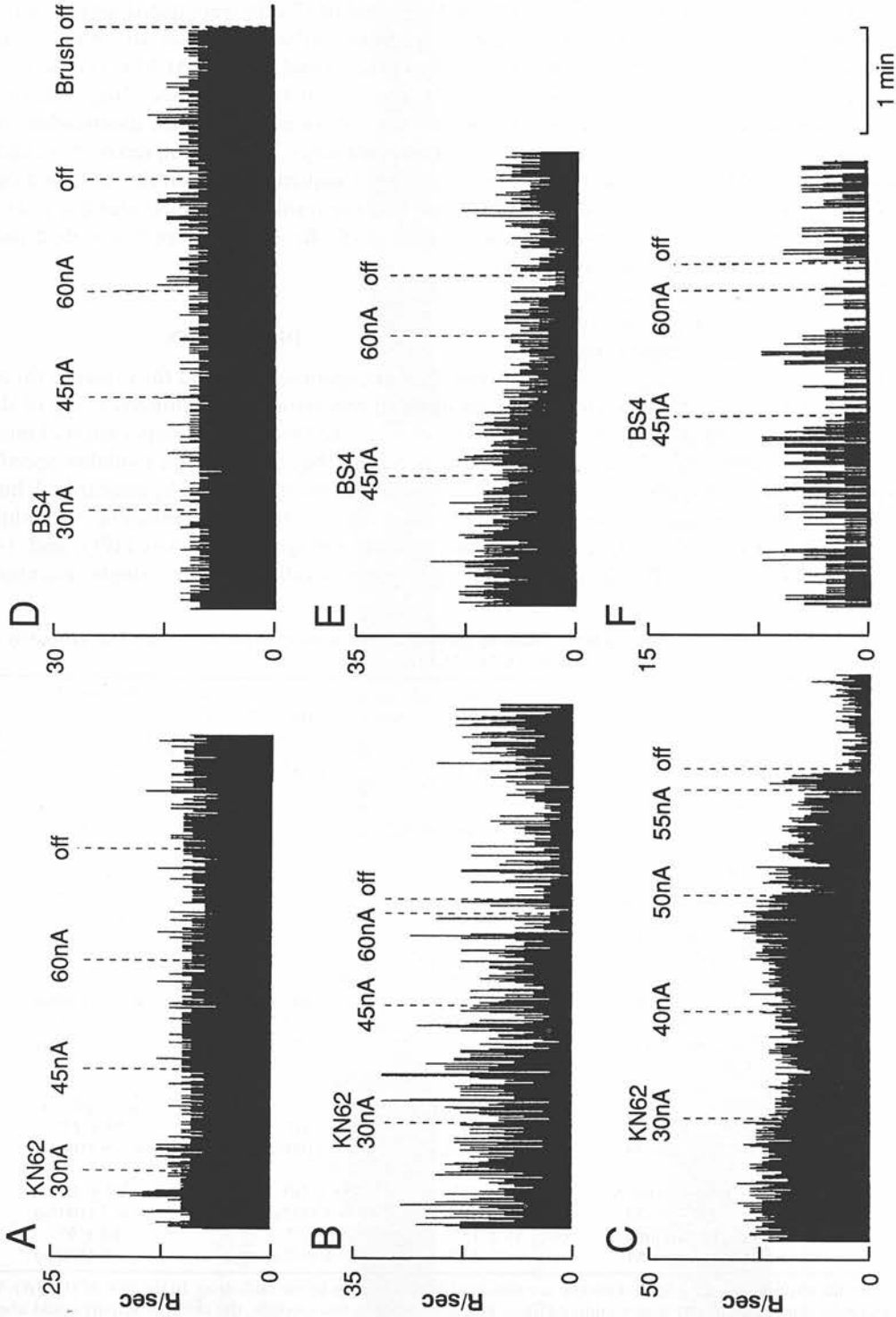


Fig. 2. Effects of ionophoretically-applied inhibitors of intracellular signalling pathways on evoked responses of dorsal horn neurons. Individual records of ongoing firing frequency are displayed as the action potentials per second (R/sec), integrated over 700 msec bins, plotted against time. (A–C) demonstrate typical effects of the  $\text{Ca}^{2+}$ /calmodulin kinase II inhibitor KN62 on brush-, mustard oil- and (1S,3R)-ACPD-evoked activity (1 nA), respectively; (D–F) show typical effects of the phospholipase  $\text{A}_2$  inhibitor BS4 on brush-, mustard oil- and (1S,3R)-ACPD-evoked activity (7 nA), respectively. The records are taken from separate neurons, but are entirely typical of the overall population in each case. In some cells, KN62 and BS4 attenuated not only mustard oil- and (1S,3R)-ACPD-evoked activity, but also brush responses.

(S)-CHPG and L-AP3, which display the greater activity as mGluR antagonists, failed to modify brush responses of the neurons. Interestingly, Eaton *et al.* (1993) have previously shown that (S)-CHPG affected nociceptive, but not non-nociceptive, responses of thalamic neurons. Neuronal activity evoked by the mGluR agonist (1S,3R)-ACPD was also significantly inhibited by (R,S)-CHPG or by L-AP3 (Fig. 1; Young *et al.*, 1994). MeCPG was not investigated in the present experiments due to the rather variable effects it exerts on dorsal horn neuron responses to mustard oil (Young *et al.*, 1994) and to dorsal root stimulation (Cao *et al.*, 1995). Although the mGluR antagonists inhibited mustard oil-evoked activity in 75% of the neurons tested here, (1S,3R)-ACPD only elicited neuronal firing in approximately 25% of cases. This apparent anomaly may be a consequence of the mustard oil-evoked synaptic input involving a number of other mediators beyond mGluRs; for example AMPA, neurokinin and perhaps NMDA receptors. Agonist stimulation of mGluRs alone, in the absence of other components, may be insufficient to regularly elicit overt neuronal firing in the recorded cell. Other explanations are of course possible.

The mGluR antagonist used here, (R,S)-CHPG, is a potent inhibitor of intracellular signalling responses elicited by the phosphoinositide-hydrolysing mGlu<sub>1</sub> receptor, but not by mGlu<sub>2</sub> or mGlu<sub>4</sub> receptors (Watkins and Collingridge, 1994). It is likely that the properties of mGlu<sub>5</sub> receptors correspond broadly to those of mGlu<sub>1</sub>, and further subtypes to mGlu<sub>2/4</sub> receptors, which suggests that the receptor subtype involved in the mGlu antagonist effects may well be mGlu<sub>1</sub> or mGlu<sub>5</sub>. Although the (R)-enantiomer of CHPG displays some NMDA receptor antagonism (Watkins and Collingridge, 1994) it can be inferred that the inhibitory effects of (R,S)-CHPG on mustard oil responses here are mainly due to mGluR blockade, since they are mimicked stereoselectively by L-AP3, but not D-AP3 (effective at mGluR and NMDA receptors respectively; Young *et al.*, 1994).

This evidence for mGluR involvement in a sensitized nociceptive response is consistent with evidence from other studies on the amplification of neuronal responses by intra-articular inflammation or carrageenan-induced Neugebauer *et al.*, 1994; Young *et al.*, 1995). It is clear, however, that mGluRs are not the sole mediator of sensitization, since both NK<sub>2</sub> and NK<sub>1</sub> neurokinin receptors in the dorsal horn can also contribute to the sustained nociceptive activation and increased neuronal responsiveness (Xu *et al.*, 1991; Munro *et al.*, 1993; Thompson *et al.*, 1994). Interestingly, the mGlu<sub>1</sub> and mGlu<sub>5</sub> subtypes of mGluR, together with NK<sub>1</sub> and NK<sub>2</sub> receptors have the common property of signal transduction by means of phosphoinositide hydrolysis (Nakajima *et al.*, 1992; Watkins and Collingridge, 1994). Correspondingly, neuronal responses to mustard oil, but not brush, were significantly reduced by inhibitors of PKC and by calmidazolium, an antagonist of the Ca<sup>2+</sup> binding protein calmodulin (thence Ca<sup>2+</sup>/calmodulin

kinase II) both classical mediators of phosphoinositide-hydrolysing receptors (Table 1). Responses to the mGluR agonist (1S,3R)-ACPD were also significantly inhibited by these reagents, consistent with the hypothesis that a phosphoinositide-hydrolysing mGluR may participate in mediating responses to mustard oil. Both Ca<sup>2+</sup>/calmodulin kinase II and the Ca<sup>2+</sup>/calmodulin-activated phosphatase calcineurin, have been implicated in long term changes in responsiveness in hippocampal neurons (Silva *et al.*, 1992; Lisman, 1989). It is not possible to draw any conclusion from the experiments here with KN62, since it significantly inhibited all types of activity tested in the overall population of cells and there is thus no assurance that its effects are other than non-specific.

The prominent blockade of mustard oil-evoked activity by PKC inhibitors (Table 1; Munro *et al.*, 1994a) is consistent with the translocation/activation of dorsal horn PKC observed in both inflammatory and loose-ligation neuropathy models of sustained nociception (Mao *et al.*, 1993; Munro *et al.*, 1994a). The sensitivity of hippocampal LTP to inhibition of PKC (Bliss and Collingridge, 1993) maintains the analogy between that phenomenon and the sensitization of dorsal horn neurons by sustained nociceptive input. Furthermore, PKC inhibitors attenuate the behavioural nociceptive responses elicited by subcutaneous formalin injection (Coderre, 1992; Coderre and Yashpal, 1994) and also attenuate the enhancement of such responses brought about by glutamate or substance P. Metabotropic glutamate, NK<sub>1</sub> and NK<sub>2</sub> receptor agonists can facilitate neuronal responses to NMDA (Cerne and Randic, 1992; Bleakman *et al.*, 1992; Rusin *et al.*, 1992) and, where tested (on the NK<sub>1</sub> agonist, substance P), these effects were reversed by a protein kinase inhibitor (Rusin *et al.*, 1992).

Not only PKC-activating phorbol esters (Gerber *et al.*, 1989) but also the PKA activator forskolin (Cerne *et al.*, 1993) can facilitate neuronal responsiveness in the dorsal horn, but the inhibitory effects of the PKA inhibitors KT5720 and H89 here are equivocal because all types of activity were inhibited and the possibility of non-specific actions cannot be excluded. No evidence was found in support of the hypothesis that the mGluR subtype here might be one acting by adenylate cyclase inhibition, in which case PKA inhibitors might have been expected to mimic rather than inhibit (1S,3R)-ACPD responses.

The two PLA<sub>2</sub> inhibitors tested, ONO-RS-082 and BS4, significantly inhibited both mustard oil and (1S,3R)-ACPD responses, whereas ONO-RS-082 had no significant effect on brush-evoked activity and BS4 showed at least twice the magnitude of effect on the other responses than on brush. These agents are reported to block low and high molecular weight species of PLA<sub>2</sub> respectively, and in each case both Ca<sup>2+</sup> and PKC have been implicated in their activation cascades (Banga *et al.*, 1986; Oinuma *et al.*, 1991; Mayer and Marshall, 1993). The present results are consistent with observations in behavioural models implicating PKC and prostanoids in

the development of hyperalgesic states (Coderre, 1992; Malmberg and Yaksh, 1992a,b; Coderre and Yashpal, 1994).

Two selective inhibitors of tyrosine kinases (which could potentially disrupt the effects of either growth factor receptor tyrosine kinases or of receptor-independent tyrosine kinases operated downstream of phosphoinositide hydrolysis responses) were also tested. These agents caused generalized inhibition of all types of evoked activity and so no clear conclusions can be drawn about their actions in the context of the present experiments.

In summary, the present results suggest that PKC, PLA<sub>2</sub> and perhaps a calmodulin-dependent mechanism, such as Ca<sup>2+</sup>/calmodulin kinase II, play a role in the mustard oil model of sustained and sensitized nociception in the dorsal horn and that an mGlu<sub>1</sub> or mGlu<sub>5</sub> receptor may be one of the synaptic mediators involved in triggering these signal transduction pathways.

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THE INTERACTION BETWEEN NEUROKININ<sub>1</sub> AND METABOTROPIC GLUTAMATE RECEPTOR ANTAGONISTS IN BEHAVIOURAL NOCICEPTIVE TESTS IN NORMAL AND CARRAGEENAN-TREATED RATS. M.R. Young, S.M. Fleetwood-Walker, P.J. Birch<sup>1</sup>, C. Bountra<sup>2</sup> and J. Bowers<sup>2</sup>, Department of Preclinical Veterinary Sciences, University of Edinburgh, U.K., <sup>1</sup>Department of Molecular Science (Greenford), <sup>2</sup>Pharmacology I (Ware), Glaxo Group Research, U.K. SPON: European Brain and Behaviour Society.

Intrathecal metabotropic glutamate receptor antagonists (S)-4-carboxy-3-hydroxy-phenylglycine [(S)-CHPG, (48nmol)] and L-1-amino-3-phosphopropionic acid (L-AP3, 60nmol), increase paw withdrawal latencies (PWLs) to both noxious mechanical and noxious thermal stimulation, in conscious rats. Such elevations were more profound after carrageenan-induced inflammation in the paw. However, in the same study the NK<sub>1</sub> receptor antagonist, GR82334 (3nmol), failed to alter PWLs (Young *et al* (1995) Br. J. Pharm. 114:316P). We have addressed the effect of combined mGlu and NK<sub>1</sub> receptor antagonists in such behavioural nociceptive tests. Lister-hooded rats (30-70g), with either normal or carrageenan-inflamed paws, received a co-injection of GR82334 and either (S)-CHPG or L-AP3 (n=16 per group), at doses equivalent to those above (in 10µl). Now, the increases in PWL due to mGluR antagonists were occluded when GR82334 was given simultaneously, except the (S)-CHPG-induced increase in PWL to noxious thermal stimulation in the non-inflamed paw. Statistical significance of effects (% change from pre-drug levels) was determined compared to mGluR antagonists alone, \*\* - p<0.01; \* - p<0.05 (Mann-Whitney U-test) w.r.t. the co-injection in the inflamed state:

		L-AP3	Co-injection	(S)-CHPG	Co-injection
MECHANICAL	1 min	123±47	19±8*	130±31	54±19**
THERMAL	1 min	97±6	9±9**	71±10	11±7**

These results indicate that in the state of carrageenan-induced hyperalgesia, NK<sub>1</sub> receptor antagonism appears to exert an adverse effect on the analgesia normally elicited by mGluR antagonists.